NATO Science for Peace and Security Series - A:
Chemistry and Biology

Advanced Bioactive Compounds Countering the Effects of Radiological, Chemical and Biological Agents

Strategies to Counter Biological Damage

Edited by
Grant N. Pierce
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Advanced Bioactive Compounds Countering the Effects of Radiological, Chemical and Biological Agents

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Series A: Chemistry and Biology

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Preface

In the modern era, the probability for humans to be exposed to radiological, chemical and adverse biological agents has increased exponentially creating a very real risk for injuries and deaths. There are a great number of examples of these dangers that we face, most of the time without any foreknowledge or preparation for the challenge to our health. The stockpiling of enormous amounts of weaponry during the Cold War without proper disposal or destruction in subsequent years has led to the potential for these agents to get into the hands of rogue regimes or terrorists. An increase in the number of contacts between people due to the growth and massive migration of populations, and the availability of more frequent and faster travel escalates the risk of contraction and transmission of potentially deadly infectious diseases. The increase in environmental pollutants through industrial emissions, municipal waste disposal, dissemination of persistent and highly carcinogenic explosives in the war zones, excessive use of fertilizers, pesticides, genetically modified organisms, etc. has been associated with an increased incidence of cardiovascular diseases (CVD), respiratory problems and cancer. Other natural and man-made ecological catastrophes, exemplified by the accidents at the Chernobyl and Fukushima stations, have brought humans in close contact with extremely dangerous radiological, chemical and biological challenges. People have undergone frequent medical treatments (electrocardiography, exercise treadmill testing, electron-beam computerized tomography, magnetic resonance imaging, radiation therapy and chemotherapy) and become extremely susceptible to the adverse effects of radiological, chemical and biological agents. Military personnel, firefighters, pilots, and medical staff are more frequently exposed to radiological, chemical, and biological agents through their normal working environment. Additionally, military personnel are often experiencing enormous psychological stress, hyper- and hypo-thermal conditions and oxygen deprivation, which lead to increased morbidity and mortality.

Understanding the mechanisms, whereby radiological, chemical and biological agents inflict damage to our bodies, is essential if we are to prepare ourselves for these challenges. Currently, there are no pharmaceutical preparations which could be prophylactically taken to reduce the effect of radiological, chemical and biological

agents. Additionally, there are no established protocols for alleviation of the adverse health effects of long-term exposure to radiological or chemical agents.

A majority of the adverse health effects induced by radiological, chemical and biological agents are caused by an increase in the production of highly reactive oxygen and nitrogen species. These strong oxidants display high reactivity with lipids, proteins and nucleic acids in the body. Ultimately, these processes all play a role in the emergence of chronic diseases like neurodegeneration, heart and respiratory disease, diabetes and cancer.

There is evidence that certain natural bioactive compounds can neutralize free radicals and non-radical oxidants manifesting beneficial health effects. For example, phenolic compounds, found in cereals, legumes, nuts, olive oil, vegetables, fruits, tea and red wine, have antioxidant and anti-inflammatory properties and demonstrate favorable effects on thrombosis, tumorogenesis, CVD, prostate and other cancer. Phytoestrogens, which are present in soy, flaxseed and other whole grains, fruits and vegetables, show beneficial effects on a variety of CVD risk factors and cancer. Anti-carcinogenic and cardioprotective effects have been demonstrated by organosulfur compounds from garlic and onions, isothiocyanates in cruciferous vegetables and monoterpenes in citrus fruits and herbs. Other bioactive compounds include saponins in legumes, terpenoids from citrus, glucosinolates in cruciferous vegetables, lignans present in flaxseed, barley, soy, berries, and other fruit/vegetables and tannins found in various plants, berries, coffee, tea, chocolate and wine. Bioactive compounds from natural sources demonstrate low toxicity, complete biodegradability, availability from renewable sources, fewer side effects and a relatively low cost. They can be taken regularly as an everyday component of the diet. This makes them an attractive alternative to conventional drugs.

The NATO Advanced Research Workshop “Advanced Bioactive Compounds Countering the Effects of Radiological, Chemical and Biological Agents” held in Yalta, Crimea, Ukraine, on May 16–19, 2012, brought together scientists and professionals from research institutes and industry to discuss the science behind the health benefits of bioactive compounds and develop the best practice tools for their science-based implementation. The organization of the ARW in Ukraine created an exciting opportunity for the participants from many different countries to exchange ideas on counteracting the adverse effects of hazardous agents. Using basic and clinical research, the participants discussed new ideas for the development of bioactive dietary products with great therapeutic potential.

The Workshop was divided into four sessions: (1) Tissue Damage due to Radiological Agents, (2) Preventing the Harmful Health Effects of Biological Agents, (3) Bioactive Compounds from Natural Sources for the Prophylaxis and Treatment of the Effects of Radiological, Chemical and Biological Agents and (4) Biotechnological and Therapeutical Aspects of Defense against Radiological, Chemical and Biological Agents. A round table on the topic “Best Practice in Conducting Clinical Trials to Identify Therapeutic Efficacy of the Bioactive Compounds” was conducted during the conference.

All of the above subjects are covered in this book. It is expected that this compilation of papers will be helpful to an international community of scientists, medical

professionals, experts and students dealing with countering the effects of radiological, chemical and biological agents. The conference also has had a long-lasting beneficial effect, as it resulted in the establishment of a network of collaborative links and personal friendships between the participants. Many exciting events involving the participants of the Workshop have resulted from the meeting including the creation of the Canadian-Italian Tissue Engineering Laboratory (CITEL), the *International Symposium on Adipobiology and Adipopharmacology in Burgas*, Bulgaria, on October 25–27, 2012, and strengthening collaborative links between St. Boniface Hospital Research Centre and Ukrainian specialists directed towards practical implementation of novel antioxidative dietary products.

The Workshop would not have been successful without the remarkable hospitality and assistance from the personnel of the Hotel Bristol, Yalta, Ukraine. The substantial sponsorship from Dr. Yuriy Ogay, Private Enterprise Ressfood, is greatly appreciated. We would also like to express our sincere gratitude to Dr. Deniz Beten, Programme Director, NATO Science for Peace and Security Programme, and her administrative assistant Ms. Alison Trapp for their constant interest, guidance and patience as we prepared this textbook.

Sincerely,
Grant N. Pierce, Volodymyr I. Mizin and Alexander Omelchenko
Winnipeg, Manitoba, Canada and Yalta, Crimea, Ukraine
The Editors

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Part I
Tissue Damage due to Radiological Agents

Chapter 1
Targeting NF-κB to Prevent Radiation-Induced Carcinogenesis

M.A. Christine Pratt

Abstract Ionizing radiation inflicts damage to cells in large part through the generation of chemical intermediates that damage DNA and generate DNA strand breaks. Radiation-induced late normal tissue toxicity is the outcome of changes in tissue following exposure to radiation that precede overt toxicity. These events include mitotic cell death especially in bone marrow and mucosal tissue, and the activation of inflammatory responses that can lead to blood vessel damage, tissue necrosis resulting from lack of oxygen and excessive extracellular matrix deposition (fibrosis). While antioxidants can prevent damage by moderating the chemistry of DNA strand breaks, other agents known as radiation mitigators can be used soon after exposure to protect essential compartments such as the bone marrow from collapse. These include agents that reinforce the rapid development of mature bone marrow and mucosa. Medical management of acute radiation syndrome following accidental exposures to ionizing radiation (IR) involves attempts to reduce the risks of infection and hemorrhage resulting from bone marrow aplasia. This involves stimulating the proliferation and differentiation of residual non-impacted or radio-resistant hematopoietic stem and progenitor cells (HSPC) with hematopoietic growth factors. Soon after irradiation radiosensitive HSPC have been shown to undergo apoptosis. It has therefore been proposed that antiapoptotic cytokines including stem cell factor, Flt-3 ligand, thrombopoietin, and interleukin-3 could be employed acutely to prevent this cell death. Moreover, acute exposure to high doses of IR induces sequential, deleterious effects responsible for a delayed multiple organ dysfunction syndrome. Of course, the caveat of preventing the death of cells with damaged DNA is carcinogenesis resulting from DNA mutations in critical genes. NF-κB constitutes a family of transcription factors best associated with

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mediating the inflammatory response. In cancer cells the activation of the inhibitor of-κB kinase (IKK) and consequently the NF-κB pathway increases resistance to ionizing radiation (IR) by facilitating cell survival, despite the presence of DNA damage and mutations. A number of small molecule inhibitors of NF-κB have been described. I discuss the potential benefit of targeting NF-κB for the prevention of radiation-induced cancers.

1.1 Therapeutics of Exposure to Ionizing Radiation

Ionizing radiation inflicts damage to cells in large part through the generation of chemical intermediates that damage DNA and generate DNA strand breaks. Radiation-induced late normal tissue toxicity is the outcome of changes in tissue following exposure to radiation that precede overt toxicity. These events include mitotic cell death especially in bone marrow and mucosal tissue, and the activation of inflammatory responses that can lead to blood vessel damage, tissue necrosis resulting from lack of oxygen and excessive extracellular matrix deposition (fibrosis). While antioxidants can prevent damage by moderating the chemistry of DNA strand breaks, other agents known as radiation mitigators can be used soon after exposure to protect essential compartments such as the bone marrow from collapse. These include agents that reinforce the rapid development of mature bone marrow and mucosa. Medical management of acute radiation syndrome following accidental exposures to ionizing radiation (IR) involves attempts to reduce the risks of infection and hemorrhage resulting from bone marrow aplasia. This involves stimulating the proliferation and differentiation of residual non-impacted or radioresistant hematopoietic stem and progenitor cells (HSPC) with hematopoietic growth factors including granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF treatment promotes rapid development of mature bone marrow and mucosa from the stem cell compartment. Colony-stimulating factor remains the therapeutic standard after exposure to less than the lethal dose 50 % (Haematopoietic [H] score 3-H3) while higher scores are treated with stem cell transplantation. Soon after irradiation radiosensitive HSPC have also been shown to undergo apoptosis [1, 2]. It has therefore been proposed that antiapoptotic cytokines including stem cell factor, Flt-3 ligand, thrombopoietin, and interleukin-3 could be rapidly employed to prevent acute cell death [1].

1.2 The NF-κB Pathway and Its Regulation by Receptors and Ionizing Radiation

Of course, the caveat of preventing the death of cells with damaged DNA or promoting their proliferation is carcinogenesis resulting from DNA mutations in critical genes. Tissue damage arising from IR results in activation of the inflammatory process.

Activated macrophages secrete cytokines including TNF-α and IL-1, both of which interact with receptors on T lymphocytes. There are two TNF receptors, I and II, which both result in activation of NF-κB and have been implicated in cancer (reviewed in [3]). NF-κB is a transcription factor that is composed of members of the Rel family including p65RelA and p105/p50 (NF-κB1) and p100/p52 (NF-κB2), RelB and c-Rel. These proteins function as heterodimers or in the case of p52 and p50, can also form homodimers to regulate target genes. The inhibitor of κB (IκBα) kinase (IKK) complex consists of IKK-α,-β and -γ (NEMO) which negatively regulates IκBα activity. The *canonical NF-κB pathway* is activated by numerous cellular stresses and inflammatory cytokines including TNF-α. In this pathway IκBα binds and retains p65 in the cytoplasm until stimulated IKK-β-mediated phosphorylation results in ubiquitin-mediated degradation of IκBα allowing p65/p50 heterodimerization and nuclear translocation (reviewed in [4, 5]). A second pathway, called the *alternative NF-κB pathway* (NF-κB AP), is usually induced by activation of specific members of the TNF receptor family including the lymphotoxin (LT)α/β, B-cell activating factor (BAFF) receptors, CD40, and receptor activator of NF-κB (RANK) and is involved in lymphoid organ development and adaptive immunity [6]. Here, activation of the NF-κB inducing kinase, NIK, activates IKK-α homodimers which phosphorylates p100 to signal partial proteolytic processing to mature p52. p52 then translocates to the nucleus to participate in transcriptional activation [4–6]. RelB associates with unprocessed p100 in the cytoplasm and its nuclear translocation occurs coordinately with p52 [7]. Homodimers of p52 are transcriptionally inactive but can also combine with Bcl-3 to mediate transactivation [8, 9].

Importantly, NF-κB can also be activated in an atypical manner by *DNA damage* (Fig. 1.1). NEMO becomes SUMOylated by PIASy following DNA damage, and then undergoes nuclear translocation where it is phosphorylated at serine 85 by the ATM DNA damage-induced kinase which stimulates cIAP1 E3 ligase-mediated mono-ubiquitination of NEMO resulting in nuclear export and subsequent activation of IKK-α and IKK-β [10].

The NF-κB alternative pathway is also activated by DNA damage as evidenced by induced p100 processing and nuclear localization of the p52 subunit in an IKK-α, ATM and NEMO-dependent manner following DNA damage [11]. While the canonical pathway has been associated with cell survival following DNA damage, the alternative pathway is reported to promote cell death.

1.3 NF-κB Induction by TNFα and Cancer Progression

Interestingly, ATM has been shown to primarily mediate cell survival since downregulation sensitizes cells to IR [12]. In cancer cells the activation of the inhibitor of κB kinase (IKK) and consequently the NF-κB pathway increases resistance to ionizing radiation (IR) by facilitating cell survival, despite the presence of DNA damage/mutations. Importantly, TNFα and IL-1 can stimulate the production of NF-κB-responsive cytokines which further induce growth

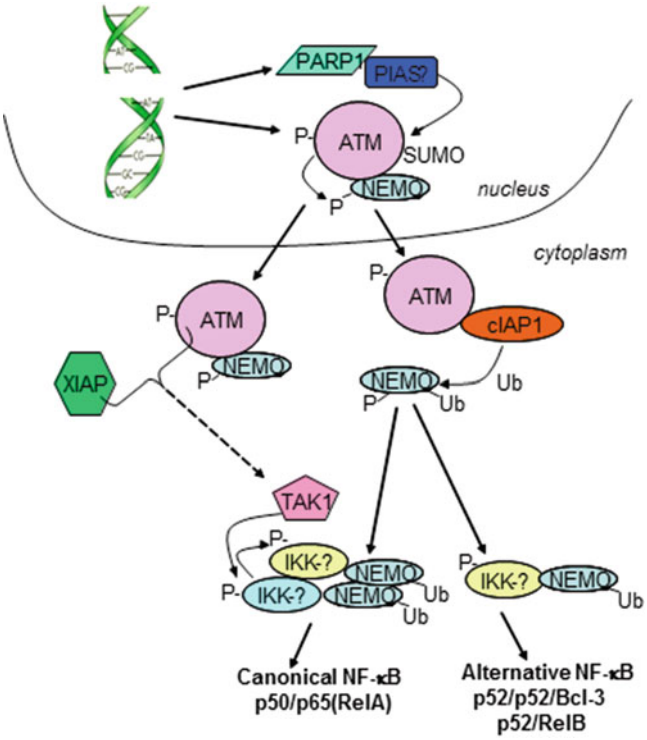

Fig. 1.1 Both the canonical and alternative NF-κB pathways can be activated following IR/DNA damage through a mechanism involving phosphorylation and signalling through the ataxia telangectasia mutated (ATM) protein

factors to promote survival and stem cell expansion. The therapeutic value of promoting bone marrow maturation from stem cells is clear promoting expansion and maturation of cells that have either escaped IR damage or those that have successfully repaired their DNA as shown in Fig. 1.2.

However, survival signalling combined with the establishment of a growth factor: cytokine feedforward pathway may allow the progression of hematopoetic stem cells harbouring cancer initiating mutations through TNF mediated signalling to NF-κB as shown in Fig. 1.3.

Clearly the risk to benefit ratio associated with replenishing the bone marrow compartment and the risk of enhancing cancer progression needs to be carefully considered. Should, for example, both high dose and very high dose radiation exposure be treated similar to multiple myeloma avoid potential expansion of an initiated population? Treatment of multiple myeloma involves induction therapy with high-dose chemotherapy to ablate the haematopoetic stem cell compartment which is then followed by autologous stem cell transplant.

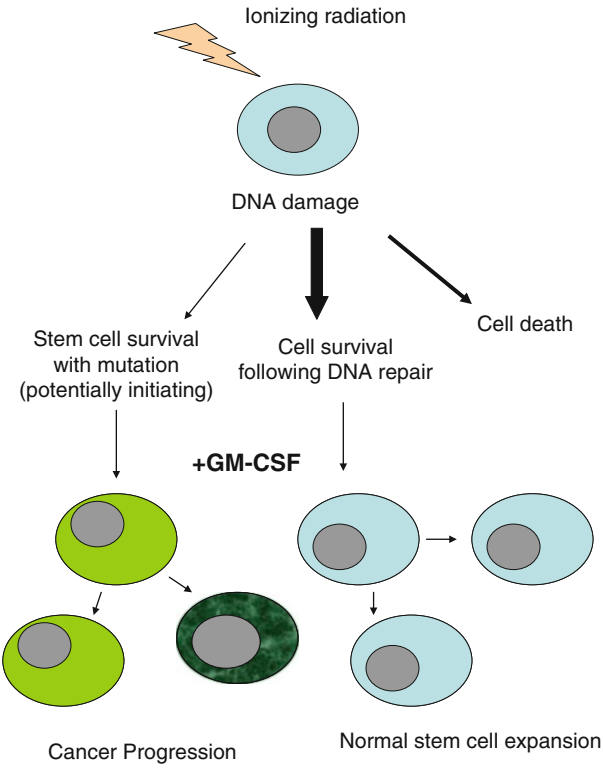

Fig. 1.2 Ionizing radiation induces DNA strand breaks in heamatopoetic stem/progenitor cells. Some cells die outright while DNA repair will render most surviving cells normal. However, repair can introduce mutations in some cells that will be expanded through therapeutic administration of GM-CSF

Maintenance and/or induction can include bortezomib and steroids [13] both of which block NF-κB to prevent further signalling through this pathway. Of course the drawbacks include that applying this approach would be difficult on a large scale and would require further induction treatment to ablate residual cells. Another option could be a more limited exposure to GM-CSF with a set minimum recovery of the haematopoetic index followed by intermittent bortemozib or steroids to block initiated cell survival and proliferation. Promotion of initiated cells might also be better prevented by measures to block inflammation arising from tissue damage which would increase the production of TNFα and IL-1 from activated macrophages. To this end, therapeutics could include factors such as keratinocyte growth factor and erythropoietin as suggested by Herodin and Drouet [1] to ensure tissue damage repair and mitigate the inflammatory processes.

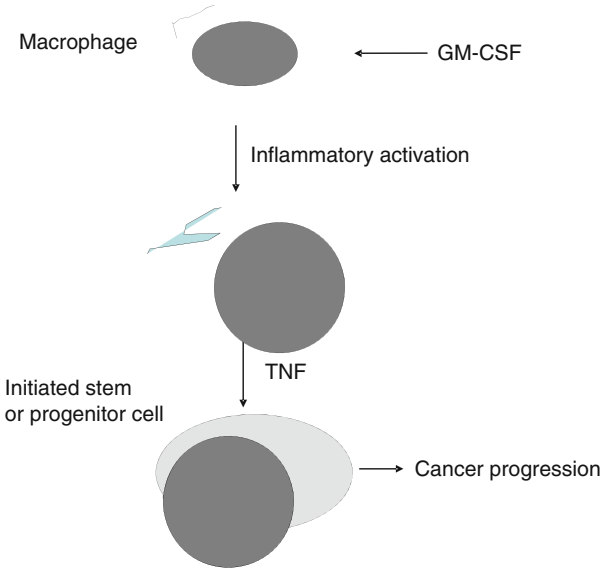

Fig. 1.3 GM-CSF induces the maturation of macrophages which can become activated as a result of tissue damage. Production of TNFα results in activation of TNFRs on lymphocytes and other cell types inducing NF-κB which can promote cancer progression

1.4 Conclusion

It is difficult to establish standard therapies for radiation exposure given the inability to run randomized clinical trials. However, our knowledge of mechanisms of long term cell survival and cancer promotion should guide in the rational treatment of high level exposure to ionizing radiation and efforts to spare damaged cells balanced with the potential for mediating cancer progression.

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Chapter 2
Antioxidants as a Bio-shield Against Radiological Weapons

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Abstract There are two types of radiological weapon, "dirty bomb," and nuclear weapon (atom bomb). A dirty bomb can be made from one or more commercially available radioactive isotopes and it can be detonated using a conventional explosive, whereas an atom bomb consists of fissionable element, and it requires complex procedures for detonation. Explosion of a radiological weapon can cause a few injuries to mass casualties, depending upon the type of radiological weapon, and can increase the chronic health risks among survivors. Bio-Shield refers to chemicals or biologics that can prevent or mitigate radiation injury when administered before and/or after irradiation. During past decades, several radiation preventive and mitigating agents been identified. They can be grouped into following categories: (a) chemicals not approved by the FDA, (b) drugs approved by the FDA for other conditions, (c) certain biologics approved by the FDA, and (d) antioxidants and herbs not requiring FDA approval. An effective bio-shield that can be recommended to humans must satisfy the following three criteria: (1) chemicals or biologics should prevent and/or mitigate radiation damage in the laboratory experiments when administered before and/or after irradiation; (2) they should show at least some evidence that they can prevent or mitigate radiation damage in humans; and (3) they at radiation preventive or radiation mitigating doses must be safe in human when administered on a short- or long-term basis. Published data

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show that most radiation preventing and mitigating chemicals, drugs or herbs satisfy only the first criterion of an effective bio-shield, whereas antioxidants satisfy all three.

2.1 Introduction

The threat of an explosion of a radiological weapon by the terrorists exists in the USA as well as in other regions of the world. In addition, the possibility of an unintentional nuclear conflict, although remote, exists as long as nations have nuclear arsenals. Explosion of a radiological weapon can cause a few to mass casualty, depending upon the type of radiological weapon, and it can increase the health risks among survivors that persist for a long time after radiation exposure. The purpose of this review is to describe the type and nature of radiological weapons, acute and long-term health risks following an explosion of a radiological weapon, advances in identifying chemicals and biologics that prevent and/or mitigate radiation injury. In addition, this review presents rationale and data to establish that antioxidants act as an effective Bio-Shield for preventing and mitigating acute and late radiation injuries in humans after irradiation.

2.2 Types and Nature of Radiological Weapons

Radiological weapons can be divided into two categories: "dirty bombs," also referred to as radiological dispersion devices (RDDs), and nuclear weapons that include atom bomb, hydrogen bomb, and neutron bomb. A dirty bomb can be made from one or more commercially available radioactive isotopes that emit γ-radiation (^{137}Cs and ^{60}Co), β-radiation (^{90}Sr and ^{131}I) or α-particles (^{235}U and ^{239}Pu), whereas a nuclear bomb such as an atom bomb consists of fissionable element such as ^{235}U or ^{239}Pu. Dirty bomb can be detonated using a conventional explosive, whereas the detonation of an atom bomb requires complex procedures, such as assembly of supercritical mass of fissionable material and injection of neutron to initiate chain reaction.

Radioactive isotopes are released following the explosion of a dirty bomb. These radioactive isotopes can travel away from the explosion site, depending on the direction and speed of the wind. They can contaminate water and food, and food sources, for a long time to come, depending upon the half-lives of dispersed radioactive isotopes. Humans living in the vicinity of the explosion can be exposed to low doses of radiation from the radioactive fallout. An explosion of an atom bomb explosion releases distinct forms of energy that include blast, shock waves, thermal radiation, ionizing radiation, radioactive fallout and electromagnetic pulse (EMP), which can cause mass destruction of buildings and infrastructures and massive human and animal casualties. The population leaving near the site of explosion can be exposed to lethal doses of ionizing radiation; others living further away from the site of explosion could be exposed to no-lethal (low) doses of radiation.

2.3 Types of Radiation Injury

Exposure to high doses of low LET (linear energy transfer) ionizing radiation, such as X-rays and γ-rays or high LET radiation such as proton and α-particles can produce acute radiation sickness (ARS) that include bone marrow (BM) syndrome, gastrointestinal (GI) syndrome and central nervous system (CNS) syndrome. High LET radiation is more damaging than low LET radiation. Low LET radiation doses of 2.5 Sievert (Sv) to 4 Sv produce BM syndrome in humans with a mortality rate ranging from a few percent to 100 % within 60 days. Doses of 6–40 Sv) produce GI syndrome with a mortality rate of 100 % within 14 days in humans [41]. Doses of above 40 Sv can produce CNS syndrome producing 100 % mortality within 24 h. Doses 0.25–2 Sv are unlikely to cause any mortality, but they can increase the risk of cancer and non-neoplastic diseases [41].

The survivors of high or low doses of radiation have increased risk of both neoplastic and non-neoplastic diseases. The increase in the risk of leukemia incidence following radiation exposure can be observed within 10 years; however, the risk of solid tumor persists for 30 years or more after irradiation. Thyroid cancer, breast cancer and hematopoietic cancer are most frequent after irradiation [13]. The primary late effects of low doses of radiation include increased risk of cancer, eye defects, birth defects (pregnant women), and somatic and heritable mutations The non-neoplastic diseases following exposure to high doses of radiation include cataract and delayed necrosis in the brain, muscle, auditory ossicles and bone [41]. In addition, the risk of somatic and heritable mutations also increases among survivors of radiation exposures [42].

2.4 Current Recommendations for Prevention and Mitigation of Radiation Damage

For prevention of radiation damage in humans, only physical principles are recommended by the national radiation agencies. These principles include physical shielding by high atomic number elements, increasing distance from the radiation sources to the recipients and decreasing radiation exposure time. The adoption of these principles can reduce the levels of radiation dose to the individuals, and thereby, reduce the tissue damage. However, it is not possible to adopt these principles under most radiation conditions, such as explosion of a radiological weapon. The physical principles of radiation protection play no role in mitigating radiation injury.

There are no approved pharmacological agents for mitigating of radiation injury except potassium iodide in humans; however, there are some approved biologics that are effective in mitigating radiation injury. These biologics are not adequate, if the radiation doses are high or if the number of individuals exposed to lethal doses of radiation is huge.

2.5 Concept of a Bio-shield

Bio-shield refers to chemicals or biologics that can protect tissue damage when administered before and/or after exposure to ionizing radiation. It includes both radioprotective agents (prevention of damage) and radiation mitigating agents (treatment of damage). An ideal Bio-Shield must satisfy the following three criteria before it can be recommended to humans for reducing radiation injury: (1) chemicals or biologics should prevent and/or mitigate radiation damage in the laboratory experiments (cell culture and animals models) when administered before and/or after irradiation; (2) they should show at least some evidence that they can prevent or mitigate radiation damage in humans; (3) they at radioprotective or radiation mitigating doses must be safe in human when administered on a short- and long-term basis. During past decades, several radioprotective and radiation mitigating agents have been identified. They are briefly described here.

2.6 Agents Preventing Radiation Injury (Radioprotective Agents)

In previous studies, most radioprotective agents were effective in reducing radiation injury only when administered before irradiation; they were ineffective when administered immediately after irradiation. They have been grouped in four categories, (a) chemicals and biologics not approved by the FDA, (b) drugs approved by the FDA for other conditions, (c) herbal extracts not requiring FDA approval, and (d) antioxidants not requiring FDA approval. They are described here.

2.6.1 Chemicals and Biologics Not Approved by the FDA

The references for this section are provided in reviews [41, 43]. The radioprotective chemicals include thiols α2-macroglobulin, substance P, tumor suppressor gene p53, kinase inhibitor (EX-Rad), inhibitors of inflammation such as (Minozac) and ethyl pyruvate, mitochondrial targeting agents such as JP4-039, cytochrome c – cardiolipin peroxidase inhibitor, triphenyl-phosphonium (TPEY-Tempo) and hemigramicidin S-conjugated 4-amoni-2,2,6,6-tetramethyl-piperidine-N-oxyl (hemi-GS-TEMPO).

The radioprotective biologics include agonists of toll-like receptor (TLR) CBLB502, a recombinant protein derivative of *Salmonella enterica* flagellin, and a synthetic lipopeptide CBLB600; a thrombopoietin agonist, Alxn4100TPO (4100TPO); cytokines , IL-12, synthokine SC-55494, a synthetic cytokine, IL-11; IL-4, IL-1 and TNF-α and hepatocyte growth factor; a somatostatin analog SOM230 (pasireotide) and SOM230-LAR, a long-acting release form of SOM230; BIO 300; and vaccines (typhoid-paratyphoid vaccine) and vaccine against Specific Radiation Determinant (SRD) [32]. These radioprotective chemicals and biologics increased the 30-day

survival rates of irradiated animals exhibiting bone marrow syndrome when administered before irradiation. These chemicals and biologics satisfy only the first criterion of an ideal Bio-Shield. Therefore, they are not suitable for human use at this time.

2.6.2 Drugs Approved by the FDA for Other Conditions

The radioprotective drugs include amifostine, an analog of cysteamine [4]; diltiazem (DTZ) [39]; angiotensin converting enzyme (ACE) inhibitors (captopril, enlapril, and fosinopril) [37]; statins such as lovastatin [40], pravastatin [17] and simvastatin [55]; and inhibitors of histone deacetylase, such as valporic acid and phenylbutyrate [10]. These radioprotective drugs increased the 30-day survival of irradiated animals (whole-body irradiation with radiation doses that produce bone marrow syndrome) when administered before irradiation. These drugs satisfy only the first criterion of an ideal Bio-Shield; therefore, they cannot be used in humans at this time.

2.6.3 Herbal Extracts Not Requiring FDA Approval

The references for this section are provided in reviews [3, 43]. Herbs are considered food supplement in the USA; therefore, their use in humans does not require FDA approval. Herbal extracts individually or in combination have been used in Asia to treat chronic human diseases in which increased oxidative stress and inflammation play a central role in the initiation and progression of the chronic disease. Several (about 24) ethanol-, methanol-, water-extracts or semi-purified herbal extracts have exhibited varying degrees of radioprotective activities in both cell culture and rodent models, when administered before irradiation with lethal doses of X-rays or γ-rays. The radioprotective herbs include an ethanol extract of *Nigella sativa*, *Acorus calamus*, *Aloe vera*, *Mentha piperita*, *Tinospora cordifolia*, *Podophyllum hexandrum* (Himalayan mayapple), *Vernonia cinerea*, *Centella asiatica*, *Ginkgo biloba*, *Hippophae rhamnoides*, *Ocimum sanctum*, *Panax ginseng*, tea polyphenols and epigallocatechin, *Psidium guajava* (common guava), *Biophytum sensitivum*, *Alstonia scholaris* (bark extract), *Emblica officinalis* (fruit extract), *Boerhaavia diffusa*, and *Phyllanthus amarus*, quercetin, genistein, curcumin, and resveratrol. At this time, herbal extracts satisfy only the first criterion of an ideal Bio-Shield; therefore, they cannot be recommended for the human use.

2.6.4 Antioxidants Not Requiring FDA Approval

Antioxidants are considered food supplements; therefore, their consumption by humans does not require FDA approval. However, pharmacological prepared and genetic engineered antioxidant enzymes will require FDA approval for use in

humans. Some antioxidants, such as antioxidant enzymes, glutathione, alpha-lipoic acid and coenzyme Q10 are endogenously made; while others, such as vitamins A, C and E, carotenoids and mineral selenium are consumed through the diet. Therefore, consumption of both dietary and endogenous antioxidants may be necessary for producing an optimal effect on prevention or mitigation of radiation damage. The studies on radioprotective efficacy of antioxidants are described here.

2.6.4.1 Cell Culture Studies with Individual Antioxidants

In 1982, we discovered that α-tocopheryl succinate (α-TS) is the most effective form of vitamin E in inducing selective death in cancer cells [44]. Later, we discovered that α-TS treatment before irradiation decreased radiation-induced chromosomal damage in normal human fibroblasts in culture, but enhanced it in human cancer cells in culture [29]. It has been shown that α-TS and selenium, but not α-tocopheryl acetate reduced radiation-induced transformation in normal mammalian cells in culture; the combination of α-TS and selenium was more effective than the individual agents [8, 45]. Other forms of vitamin E such as α-tocopherol and α-tocopheryl acetate, vitamin C and β-carotene inhibited radiation-induced mutations, chromosomal damage and lethality in mammalian cells in culture [26, 53, 56]. γ-Tocotrienol and pentoxifylline (improved local blood flow to the intestine) protected radiation induced oxidative stress in the intestine when administered before whole-body irradiation with a dose of 12 Gy that produces GI syndrome. γ-tocotrienol was more effective than that produced by the combination of γ-tocotrienol and pentoxifylline [11]. Natural β-carotene was more effective than the synthetic one in reducing radiation-induced transformation in mammalian cells in culture [21]. Treatment of lymphocytes in culture with lycopene before irradiation with 1, 2, and 4 Gy significantly decreased the frequency of micronuclei formation, dicentric and translocation types of chromosomal aberrations compared to irradiation control cells. In addition, lycopene treatment of lymphocytes before irradiation decreased the levels of thiobarbituric acid reactive substances (TBARS) and hydroperoxides, and increased the activities of antioxidant enzymes, such as SOD, catalase and glutathione peroxidase, and the level of glutathione [51]. Pre-treatment of lymphocytes in culture with other antioxidants, such as n-acetylcysteine, glutathione and thioproline before irradiation with doses of 2–4 Gy also produced similar protective effects [52]. N-acetylcysteine (NAC) also attenuated radiation-induced toxicity in mammalian cells in culture [57].

2.6.4.2 Cell Culture Studies with Multiple Antioxidants

Pretreatment of human breast epithelial cells and human thyroid cells in culture with a mixture of soy-derived Bowman-Birk inhibitor (BBI), ascorbic acid, and coenzyme Q10, selenomethionine and α-tocopheryl succinate protected against space

radiation high LET radiation)-induced cytotoxicity and transformation in these cells [23]. It has been reported that the treatment of human thyroid epithelial cells in culture with selenomethionine alone also protected against space radiation-induced increase in oxidative stress, cytotoxicity and cell transformation possibly by enhancing DNA repair machinery in irradiated cells [22]. These studies suggest that the early radiobiology concept that the effect of high LET radiation on cells cannot be modified by chemical agents is no more valid.

2.6.4.3 Animal Studies with Individual Antioxidants

Several studies revealed that a single intraperitoneal (i.p.) or subcutaneous (s.c.) administrations of individual dietary or endogenous antioxidants before whole-body γ-irradiation with high radiation doses enhanced the survival rate in varying degrees in rodents [43]. Intraperitoneal administration of vitamin E and L-carnitine individually before irradiation markedly reduced radiation-induced cataract formation in rats [25]. Pre-irradiation treatment of rats with vitamin E or L-carnitine alone significantly reduced severity of brain and retinal damage in rats; however, the combination of two did not provide an additive protective effect [49], suggesting that they were protecting radiation damage by a similar mechanism. It has been reported that i.p. administration of L-carnitine reduced γ-radiation-induced cochlear damage in guinea pigs [2]. Vitamin E administered i.p. before irradiation reduced radiation-induced damage to salivary glands [46]. Retinoic acid when administered i.p. daily for 5 days before whole-body irradiation with a dose of 9–16 Gy increased the survival of intestinal crypt cells. The Do was increased from 1.30 to 1.85 Gy. [47]. Vitamin C (ascorbic acid) administered i.p. shortly before whole-body irradiation with a dose of 10 Gy increased the 30-day survival from 0 to 33 %. Treatment of mice with ascorbic acid before irradiation also improved the healing of radiation-induced wounds [19]. Treatment with vitamin E and PTX reduced radiation-induced myocardial fibrosis and improved left ventricular function in both experimental groups of animals in comparison to irradiated control animals [7].

Δ-tocotrienol when administered 24 h before or 6 hours after whole-body irradiation with a dose of 8.75 Gy provided significant protection against radiation-induced damage to hematopoietic tissue [58]. The DRF value for tocotrienol was 1.29 [15]. In another study, administration of γ-tocotrienol 24 h before and pentoxifylline 15 min before whole-body irradiation with a dose of 8 Gy was more effective in providing protection against bone marrow syndrome than γ-tocotrienol alone. The DRF value for the combined treatment with γ-tocotrienol and PTX was 1.45, whereas it was only 1.3 for γ-tocotrienol treatment alone [28].

N-acetylcysteine (NAC) increases the intracellular level of glutathione. Daily administration of NAC s.c. daily for 7 days and then 4 h before or 2 h after abdominal irradiation with a dose of 20 Gy improved 10- and 30-day survival from 5 % (irradiated control group) to greater than 50 % (NAC treated group). It also reduced the loss of villi from the irradiated small intestine and suppressed

oxidative stress in non-irradiated bone marrow [20]. Pre-treatment of mice with NAC attenuated radiation-induced liver injury by reducing free radical-mediated oxidative damage [31]. Injection of α-lipoic acid i.p. before irradiation markedly reduced cognitive dysfunction and oxidative stress. In addition, irradiated mice treated with α-lipoic acid showed intact structure of cerebellum, higher counts of intact Purkinje cells and granular cells in comparison to irradiated animals that did not receive α-lipoic acid [34]. Administration of natural β-carotene through diet daily for 1 week before whole-body irradiation with a dose of 4 Gy provided significant protection against radiation damage [5]. Pycnogenol when administered orally to rats before irradiation with a dose of 15 Gy of X-rays significantly preserved the height and number of villi, suggesting that this antioxidant can protect intestinal mucosa [12].

2.6.4.4 Animal Studies with Multiple Antioxidants

The efficacy of antioxidant mixture acting as preventive Bio-Shield on animal models (primarily rodents) irradiated with high doses of high LET radiation. For example, irradiation of mice with space radiation, a high LET radiation, increased oxidative stress; and this effect of space irradiation was reduced by pretreatment with dietary supplementation containing Bowman-Birk Inhibitor Concentrate (BBIC), L-selenomethionine or a mixture of n-acetylcysteine, sodium ascorbate, coenzyme Q10, α-lipoic acid, L-selenomethionine and α-tocopheryl succinate [16]. α-Lipoic acid administered i.p. before whole-body irradiation significantly attenuated high LET radiation (^{56}Fe-beams)-induced radiation damage, such as impairment in the reference memory, apoptotic damage in the cerebellum, and increase in DNA and markers of oxidative damage [33]. Pre-treatment of mice with multiple dietary and endogenous antioxidants before whole-body γ-irradiation with a dose of 8.5 Gy γ-irradiation increased the survival rate of irradiated animals from 0 to 40 % [43]. A few studies have shown that a mixture of dietary antioxidants administered i.p. before irradiation reduced radiation-induced myelosuppression and oxidative stress in rodents [16]. They were ineffective when injected i.p. immediately after irradiation. An oral administration of antioxidants before or after whole body x-irradiation was ineffective. In collaboration with Dr Jones of NASA at Houston, TX, it was demonstrated that an oral administration of the preparation of antioxidants as above daily for 7 days before and daily for 7 days after irradiation increased the survival time of irradiated sheep exhibiting GI syndrome associated with mild CNS syndrome from 7 to 38 days without any supportive care [43]. In collaboration with Dr. Sharmila Bhattacharya of NASA, Moffat Field, CA, it was demonstrated that dietary supplementation with the same antioxidant mixture 7 days daily before and for the entire observation period after irradiation, prevented proton radiation-induced cancer in female fruit flies carrying mutant HOP (TUM-1) that make them very sensitive to develop cancer. To our knowledge, this is a first demonstration in which the genetic basis of a disease can be prevented by multiple antioxidants.

2.6.4.5 Antioxidant Enzymes

The references for this section are described in a review [43]. It has been shown that overexpression of manganese-superoxide dismutase (MnSOD) protected hematopoietic progenitor cells against radiation damage. Overexpression of CuZnSOD or MnSOD in human primary lung fibroblasts increased the survival of irradiated cells exposed to doses of 1–6 Gy. A SOD mimetic, M40403, when administered at a dose of 40 mg/kg of body weight 30 minutes before whole-body irradiation with a dose of 8.5 Gy increased the survival from 0 to 100 %. The DRF of M40403 when administered at a dose of 30 mg/kg of body weight s.c 30 minutes before irradiation was 1.41. Overexpression of extracellular SOD in transgenic mice protected against radiation-induced lung injury. A metalloporphyrin-based SOD mimetic, MnTnHex-2-PyP5+ (hexyl), when administered at a dose of 0.05 mg/kg of body weight 2 h after irradiation reduced radiation injury to the lung in rodents. Administration of hexyl s.c. at a dose of 0.05 mg/kg of body weight daily for the first two months after thorax irradiation with 10 Gy also mitigated radiation-induced lung injury in Rhesus monkeys. There was no demonstrable toxicity of this agent. Supportive care was given as needed to these animals. Hexyl, a powerful antioxidant, when administered s.c. at a dose of 6 mg/kg of body weight 6 h after whole-body irradiation with a sublethal dose of 6.5 Gy, reduced bone marrow injury by inhibiting oxidative stress and senescence. Administration of SOD-plasmid liposome (MnSOD-PL) provided local radiation protection to the lung, esophagus, oral cavity, urinary bladder and intestine when administered before γ-irradiation. Administration of polyethylene glycol (PEGylated) antioxidant enzymes (1:1 mixture of PEG-catalase and PEG-SOD) at a dose of 100 μg i.v. before whole-thorax irradiation with a dose of 13.5 Gy reduced radiation-induced pulmonary fibrosis in mice. The use of SOD mimetic and liposomal encapsulated MNSOD satisfies only the first criterion of an effective Bio-shield; therefore, they cannot be recommended for human use.

2.6.4.6 Human Studies with Individual or Multiple Antioxidants

Administration of β-carotene orally reduced the severity of radiation-induced mucositis during radiation therapy of the head and neck cancer without affecting the efficacy of therapy [35]. A combination of dietary antioxidants was more effective in protecting normal tissue during radiation therapy than the individual agents [18, 30]. Vitamin A and NAC may be effective against radiation-induced cancer [50]. α-Lipoic acid treatment alone for 28 days lowered lipid peroxidation among children chronically exposed to low doses of radiation daily in the area of contaminated by the radioactive isotopes released following the Chernobyl nuclear power accident [27]. In another study involving 709 children (324 boys and 385 girls) who had been exposed to low levels of radiation during and after the Chernobyl nuclear power plant accident and moved to Israel between 1990 and 1994, the effect of daily natural β-carotene supplementation (40 mg/day) for a period of 3 months on the blood level of oxidative damage was evaluated. The results showed that the blood

levels of oxidized conjugated dienes in 262 children were increased compared to those who did not receive radiation. After a 3-month supplementation with β-carotene alone, the serum levels of this biomarker of oxidative damage was decreased without any significant changes in the level of total carotenoids, retinol, or α-tocopherol in these children [6]. A combination of vitamin E and α-lipoic acid was more effective than the individual agents [27]. In a randomized study involving 91 patients with lung cancer, it was demonstrated that the treatment with vitamin E and PTX markedly reduced radiation-induced lung toxicity [36]. A well-designed clinical study has reported that daily oral supplementation with curcumin, an antioxidant, during the entire course of treatment significantly reduced the severity of radiation dermatitis in breast cancer patients [48].

Human peripheral lymphocytes when irradiated with a dose 10 mGy (10 mSv) (equivalent to a dose delivered to patients in a single CT scan) showed increased number of cells with DNA double-stranded breaks (DSBs) compared to unirradiated control cells [14]. The DNA DSBs, if not prevented or repaired, can lead to neoplastic diseases. Using a commercial patented preparation of multiple dietary and endogenous antioxidants referred to as Bio-shield-R1, it was demonstrated [14] that pretreatment of human peripheral lymphocytes in culture before irradiation with Bio-shiled-R1 markedly reduced the number of cells with DNA DSBs. Bio-Shield- R1 was ineffective when administered after irradiation. In another experiment, normal individuals were given Bio-Shield orally for a period of 15, 30 and 60 min after which the peripheral lymphocytes were irradiated with 10 mGy, and then the number of cells with DNA DSBs was determined. The results showed that Bio-Shield-R1 treatment before irradiation reduced DNA DSBs by about 52 %. These limited studies suggest that dietary and endogenous antioxidants reduce radiation injury in humans. Antioxidants are also non-toxic in humans when consumed for a short or long period of time. These studies suggest that multiple antioxidants satisfy all three criteria of an effective Bio-Shield; therefore, can be recommended for human use.

2.7 Chemicals and Biologics That Mitigate Radiation Injury

2.7.1 Chemicals and Biologics Not Approved by the FDA

The references for this section are provided in a review [43]. These radiation mitigating chemicals include kinase inhibitor (EX-Rad), inhibitors of inflammation such as (Minozac) and ethyl pyruvate (EP), mitochondrial targeting agents, such as (JP4-039), cytochrome c – cardiolipin peroxidase inhibitor, triphenyl-phosphonium (TPEY-Tempo), hemigramicidin S-conjugated 4-amoni-2,2,6,6-tetramethyl-piperidine-N-oxyl (hemi-GS-TEMPO).

The radiation mitigating biologics include agonists of toll-like receptor (TLR) CBLB502, a recombinant protein derivative of *Salmonella enterica* flagellin, and a synthetic lipopeptide CBLB600; a thrombopoietin agonist, Alxn4100TPO (4100TPO);

cytokines, IL-12, synthokine SC-55494, a synthetic cytokine, IL-11, IL-1and TNF-α and hepatocyte growth factor; a somatostatin analog SOM230 (pasireotide) and SOM230-LAR, a long-acting release form of SOM230; and BIO 300. These chemicals when administered after irradiation increased the 30-day survival of irradiated animals. These chemicals and biologics satisfy only the first criterion of an ideal Bio-shield.

2.7.2 Drugs Approved by the FDA for Other Conditions

These drugs include angiotensin converting enzyme inhibitors and statins. They provide significant tissue protection when administered after irradiation. The efficacy of these radioprotective drugs in reducing radiation damage was tested primarily on rodents (mice and rats). It is unknown whether these drugs would act as a mitigating Bio-Shield at doses that are safe in humans when consumed orally on a short- or long-term basis. These drugs satisfy only the first criterion of an ideal Bio-Shield; therefore, they cannot be used in humans for mitigating radiation injury at this time.

2.7.3 Antioxidants Not Requiring FDA Approval

Since antioxidants neutralize free radicals and inhibit inflammation, and since these two biological events play a central role in the initiation and progression of radiation damage after irradiation, the use of multiple antioxidants after irradiation appears to be one of the rational choices for preventing as well as mitigating acute radiation sickness. Indeed, a recent study suggests that dietary supplementation with a mixture of antioxidants (sodium ascorbate, N-acetylcysteine, α-lipoic acid, α-tocopheryl succinate, coenzyme Q10, and L-selenomethionine) mitigated ARS in mice when administered after irradiation; however, the time of initiation of post-irradiation treatment was critical in obtaining an optimal protection [9]. Animal were fed diet rich in antioxidants immediately, 12, 24 and 48 h after whole-body irradiation with γ-rays dose of 8 Gy, and the % survival was determined 30 days after irradiation. The results showed that that the survival was 0 % in irradiated control animals or in those animals receiving antioxidant-rich diet immediately after irradiation. However, about 29, 78, and 20 % of irradiated animals receiving antioxidant-rich diet 12, 24 and 48 h after irradiation survived. These results suggest that initiation of antioxidant-rich diet 24 h after irradiation provided an optimal radiation protection in mice. The exact reasons for this phenomenon are unknown. However, the authors have suggested that delaying the start of antioxidant-rich diet 24 h after irradiation allows the repair process to progress, yielding the highest increase in survival of bone marrow cells. There could be another explanation. I suggest that immediately after irradiation, pro- and anti-inflammatory cytokines are released in response to cellular injuries.

At this time, the proportional of anti-inflammatory cytokines responsible for repair may be higher than those of pro-inflammatory cytokines responsible for the progression of damage the irradiated cells. Inhibition of inflammation soon after irradiation may prevent the release of both anti- and pro-inflammatory cytokines; and thereby can prevent the repair of radiation injury. On the other hand, by about 24 h after irradiation, pro-inflammatory cytokines dominate. Thus, it is important not to start antioxidant therapy soon after irradiation.

2.7.4 Biologics Approved by the FDA That Mitigate Radiation Injury

They include agents used in replacement therapy, erythropoietin, and bone marrow and new born liver cells. The efficacy of these biologics is described here.

2.7.4.1 Replacement Therapy

Replacement therapy includes biologics, such as antibiotics, electrolytes, platelet and whole-blood and is administered after irradiation with lethal doses of radiation. Replacement therapy is mostly effective against radiation doses that produce 60 LD_{50} in humans. This therapy can safely be applied to humans after irradiation. It should be mentioned that the replacement therapy requires large amounts of each of the biologics for the treatment of irradiated individuals; therefore, it is only suitable for the management of small number of lethally irradiated individuals. This therapy may not be able to provide adequate amounts of biologics in case of managing mass casualty.

Replacement therapy is ineffective in reducing mortality in irradiated humans exposed to radiation doses that cause GI syndrome. The best result one can hope for from the replacement therapy is increase in survival time by about twofold (from 14 to 28 days). Therefore, additional treatment modalities must be added in order to improve the efficacy of replacement therapy in management of irradiated individuals exhibiting GI syndrome.

2.7.4.2 Erythropoietin

The survival of mice irradiated whole-body with a dose of 10 Gy increased from 0 to 40 % when administered after irradiation [38]. The DRF value of erythropoietin dose (10 units) injected 1 h after whole-body irradiation with a dose of 6.51 Gy is 1.12 [54]. The efficacy of erythropoietin in irradiated individual has not been evaluated, but it is a FDA approved drug that is used in certain human diseases to boost hemoglobin levels; therefore, can be used as a radiation mitigating agent in combination with other therapeutic modalities.

2.7.4.3 Bone Marrow Cells

Bone marrow transplant is often administered intravenously to irradiated individuals exposed to doses of radiation that produce GI syndrome, because replacement therapy is not effective in increasing the survival of irradiated individuals. On the other hand, bone marrow transplantation can save many irradiated individuals dying from GI syndrome; however, the survivors die within a few years because of host vs. graft rejection events. Therefore, bone marrow transplant cannot be considered suitable treatment option for those individuals exhibiting radiation-induced GI syndrome. Isologous bone marrow cells can be used without the fear of rejection, but they are not available.

2.8 Scientific Rationale for Using Multiple Antioxidants

The rationales for using multiple antioxidants in radiation protection studies are described here. The references for this section are described in a review [43]. Most clinical studies with a single antioxidant, such as synthetic beta-carotene or vitamin E increased the risk of cancer in high risk populations [1, 24]. The mechanisms of action of antioxidants and their distribution at the cellular and organ levels differ, their internal cellular and organ environments (oxygenation, aqueous and lipid components) differ, and their affinity for various types of free radicals differ. For example, β-carotene (BC) is more effective in quenching oxygen radicals than most other antioxidants. BC can perform certain biological functions that cannot be produced by its metabolite vitamin A, and vice versa. It has been reported that BC treatment enhances the expression of the connexin gene which codes for a gap junction protein in mammalian fibroblasts in culture, whereas vitamin A treatment does not produce such an effect. Vitamin A can induce differentiation in certain normal and cancer cells, whereas BC and other carotenoids do not. Thus, BC and vitamin A have, in part, different biological functions. Therefore, both BC and vitamin A should be added to a multiple micronutrient preparation.

The gradient of oxygen pressure varies within the cells. Some antioxidants, such as vitamin E, are more effective as quenchers of free radicals in reduced oxygen pressure, whereas BC and vitamin A are more effective in higher atmospheric pressures. Vitamin C is necessary to protect cellular components in aqueous environments, whereas carotenoids, vitamins A and vitamin E protect cellular components in lipid environments. Vitamin C also plays an important role in maintaining cellular levels of vitamin E by recycling vitamin E radical (oxidized) to the reduced (antioxidant) form. The form of vitamin E used in any multiple micronutrient preparation is important. It has been established that d-α-TS is the most effective form of vitamin both in vitro and in vivo. This form of vitamin E is more soluble than α-tocopherol and enters cells more readily. It has some unique effects that cannot be produced by α-tocopherol. Therefore, for an optimal effect of vitamin E, both α-TS and α-tocopherol or α-tocopheryl acetate should be added to a micronutrient preparation.

Glutathione is effective in catabolizing H_2O_2 and anions. However, an oral supplementation with glutathione failed to significantly increase the plasma levels of glutathione in human subjects, suggesting that this tripeptide is completely hydrolyzed in the G.I. tract. Therefore, both N-acetylcysteine and α-lipoic acid that increase the cellular levels of glutathione by different mechanisms should be added to a multiple micronutrient preparation. Other endogenous antioxidants, coenzyme Q10, may also have some potential value in radiation protection. A study has shown that ubiquinol (coenzyme Q10) scavenges peroxy radicals faster than α-tocopherol, and like vitamin C, can regenerate vitamin E in a redox cycle. Selenium is a co-factor of glutathione peroxidase, and Se-glutathione peroxidase increases the intracellular level of glutathione that is a powerful antioxidant. There may be some other mechanisms of action of selenium. Therefore, selenium and coenzyme Q10 should be added to a multiple micronutrient preparation for an optimal radiation protection.

2.9 Scientific Rationale for Administering Antioxidants Before and After Irradiation for Prevention of Radiation Damage

Free radicals generated during irradiation initiate radiation injury. Long-lived free radicals and pro-inflammatory cytokines after irradiation contribute to progression of radiation damage; therefore it is essential to treat with antioxidants before or after irradiation for the entire observation period in order to produce an optimal protection against radiation damage. Pre-irradiation treatment period may include daily for 3–7 days both for animals and humans in order to increase the tissue levels of antioxidants optimally before irradiation, and post-irradiation treatment may not start until 24 h after irradiation, and then continue daily for the entire observation period.

2.10 Proposed Antioxidants as an Ideal Preventive Bio-shield Against Radiation Injury in Humans

A multiple micronutrient preparation containing preventive doses of vitamin A (retinyl palmitate), vitamin E (both d-α-tocopherol and d-α-TS), natural mixed carotenoids, vitamin C (calcium ascorbate), and coenzyme Q10, R-α-lipoic acid, n-acetylcysteine, L-carnitine, vitamin D, all B-vitamins, selenium, zinc, and chromium is proposed for reducing radiation damage. No iron, copper, manganese or heavy metals, such as vanadium and molybdenum are included, because these trace minerals are known to interact with vitamin C to produce free radicals, and heavy metals are neurotoxic. Taking this micronutrient preparation before irradiation with lethal doses of X-rays or γ-rays that produce bone marrow syndrome may reduce the symptoms of acute radiation sickness (ARS) may increase the chances of survival. The standard therapy (replacement therapy, growth factors, cell transplant

when indicated) should be provided after irradiation in order to further improve the survival rates of lethally exposed individuals. Post-irradiation treatment with antioxidants may start 24 h after irradiation and continue for the entire lifespan in order to reduce the late adverse health effects of radiation. This micronutrient strategy when combined with standard therapy may improve the clinical outcomes of irradiated individuals exhibiting GI syndrome.

2.11 Proposed Antioxidants as an Ideal Mitigating Bio-shield in Combination with Standard Therapy Against Radiation Injury in Humans

A preparation of micronutrient containing therapeutic doses of dietary and endogenous antioxidants is proposed for mitigating of radiation injury. This micronutrient preparation is administered orally 24 h after irradiation and continues twice a day (morning and afternoon with mal) for 60 days in order to mitigate ARS associated with bone marrow syndrome. The rationale for selecting post-irradiation period of 24 h has been described earlier. The antioxidant treatment period of 60 days after irradiation was selected because humans exposed to doses that produce bone marrow syndrome die within this period and those who are exposed to doses that produce GI syndrome die within 14 days. In addition to micronutrient treatment, standard therapy (replacement therapy when indicated) should be provided to all irradiated individuals exhibiting bone marrow syndrome or GI syndrome. It is expected that the proposed combination therapy may increase the survival rates of irradiated individuals exhibiting GI syndrome. A study using proposed micronutrient preparation in combination with standard therapy should be initiated to determine the efficacy of this strategy on the criteria of survival rate and survival time in animals irradiated with doses of radiation that produce bone marrow syndrome and GI syndrome.

Since survivors of high doses of radiation and those receiving low doses of radiation have increased risk of neoplastic and non-neoplastic diseases. It is recommended that administration of a preventive Bio-shield orally twice daily for the remainder of lifespan may reduce the risk of late adverse health effects of radiation.

2.12 Conclusion

Radiological weapon can be in the form of a dirty bomb or a nuclear bomb (atom bomb). A dirty bomb consists of radioactive isotopes, whereas an atom bomb requires fissionable materials such as ^{235}U. An explosion of a dirty bomb causes a few causalities, but can contaminate large surface area with radioactive isotopes, whereas an explosion of an atom bomb causes mass casualties and massive destruction of infrastructures as well as contaminates large surface area with radioactive isotopes. Doses between 2.5 to

4 Sv produce bone marrow syndrome, 6 to 40 Sv cause gastrointestinal syndrome and above 40 Sv produce central nervous system syndrome. Late adverse health effects of radiation include neoplastic and non-neoplastic diseases, and somatic and heritable mutations. Several chemicals and biologic have been identified as a radiation preventing and radiation mitigating Bio-shield, but only antioxidants can be considered as an ideal Bio-shield, because they prevent and mitigate radiation injury in laboratory experiments and in humans, and they are considered safe for human use.

Conflict of Interest The author is an employee of Premier Micronutrient Corporation, and has a financial interest.

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Chapter 3
Sensing Mechanisms of the Low-Power Infrared Radiation

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Abstract Mechanisms of interaction between low-power CO_2-laser infrared (IR) radiation and sensory neuron were investigated. The primary event of this interaction is energy absorption by Na^+,K^+-ATPase-bound ATP molecules. The subsequent transfer of vibrational energy from excited ATP molecules to Na^+,K^+-ATPase activates the enzyme converting it to a signal transducer, which results in a decrease in $Na_v1.8$ channels voltage sensitivity. The mechanism of interaction between non-thermal, low-power IR radiation and nociceptive neuron membrane is suggested. It is governed by an extremely sensitive transducer-coupled decrease in effective charge transfer in the activation gating machinery of $Na_v1.8$ channels. The Almers' method of effective charge transfer evaluation was shown to be adequate only for slow sodium channels, which have inactivation kinetics that are sufficiently slow. Another temperature-dependent mechanism of IR radiation interaction with sensory membrane is proposed due to an investigation of the behavior of $Na_v1.8$ channels inactivation. The thermal effects were clearly detected, as the duration of the sodium current trace decreased after irradiation. This phenomenon is caused by acceleration of the inactivation process. Both fast and slow inactivation time constants significantly decreased after irradiation. The activation gating system of $Na_v1.8$ channel can serve as a sensor of non-thermal radiation. This channel is also demonstrated to sense higher thermal energy by its inactivation gating machinery. The range between non-thermal and thermal thresholds is very narrow (the energies of ~200 to ~2,000 photons emitted by CO_2-laser within our patch-clamp experimental setup). A fundamental existence of this range makes it possible to predict the analgesic effect of IR

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radiation in humans, since the excitability of nociceptive cutaneous afferents is decreased exclusively within these energy thresholds. Therefore, a novel medical device, a low-power CO_2-laser for physiotherapy, was constructed.

3.1 Introduction

Low-power laser therapy has been successfully applied in clinical practice for the treatment of various pathologies. However, the molecular mechanisms of interaction between low-power infrared (IR) radiation and the sensory neuron remain rather unclear, especially those involving the radiation of mid- and far IR range [11]. Some haematocryal animals are known to perceive low-power laser radiation, in particular, CO_2-laser radiation [17], though the molecular mechanism for the detection of infrared signals is obscure [7].

A novel approach to investigate the molecular mechanism of interaction between low-power, middle IR CO_2-laser radiation and rat sensory neuron membrane has been suggested [22]. The essential feature of this mechanism is that the activation gating system of slow sodium channels ($Na_v1.8$) is highly sensitive to the action of low-power IR radiation. The sensitivity is governed by a transducer-mediated mechanism [14], which reduces the membrane excitability due to a decrease in voltage-sensitivity of $Na_v1.8$ channel activation gating system. It is well known that these channels provide the coding of nociceptive information [6]. Modulation of the effective charge, transferred upon the opening of the activation gates, is of particular significance, since these gates define a very low perception threshold of non-thermal IR radiation. The existence of the "temperature" (thermal) threshold, above which the temperature of the membrane increases, is caused by the sensitivity of the $Na_v1.8$ channel inactivation gating system. The present work is aimed to propose a mechanism of interaction between CO_2-laser radiation and sensory neurons: to find an appropriate molecular target, which could be responsible for radiation energy absorption, to suggest a pathway of the further energy utilization, and to determine which cascade processes are triggered by the absorbed energy.

3.2 Methods

Patch-clamp technique. Experiments were performed on the short-term cultured dorsal root ganglia neurons isolated from newborn Wistar rats. These nociceptive neurons are the small dark cells with a high density of $Na_v1.8$ channels [5]. Dorsal ganglia were isolated from L_5-S_1 region of the spinal cord and were placed in Hank's solution. Enzymatic treatment [13] was performed for 8 min at 37 °C in a solution containing 1 ml Hank's solution, 1 ml Eagle's medium, 2 mg/ml type 1A collagenase, and 1 mg/ml pronase E. The buffer used was 1 mM HEPES Na, pH 7.4. After this procedure, the ganglia were thoroughly washed by centrifugation (1 min, 900 rpm)

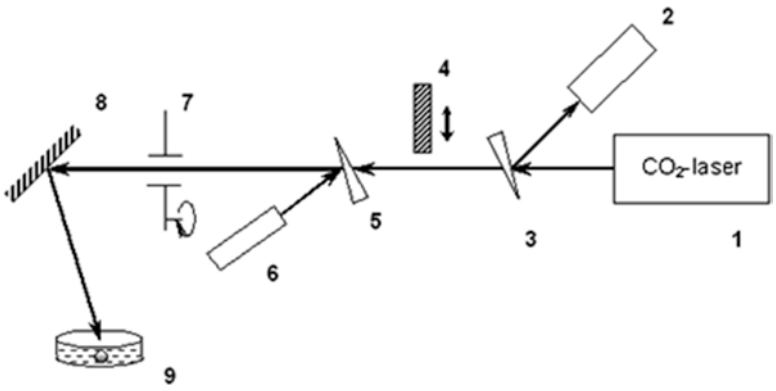

Fig. 3.1 The optical scheme for IR irradiation of nociceptive neuron. *1* – CO_2-laser; *2* – power meter; *3*, *5* – optical wedges; *4* – mechanical screen; *6* – semi-conductor laser; *7* – disc attenuator; *8* – mirror; *9* – experimental bath

with changes of the supernatant solution. Both washing and cultivation were performed using the solution consisting of Eagle's medium with glutamine based on Earle's solution (1:1), embryo calf serum (10 %), glucose (0.6 %), and gentamicin (40 U/ml). Mechanical dissociation was carried out by pipetting. The culture fluid was added to the resulting cell suspension to obtain the desired cell density in a plastic Petri dish. The non-neuronal cells were removed by allowing them to settle onto the surfaces of plastic 60-mm Petri dishes for 25 min at 37 °C, while the remaining cells, which were mostly the spinal ganglion neurons, were cultured on collagen-coated surfaces of 40-mm Petri dishes.

The extracellular solution contained 65 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 70 mM choline chloride, 10 mM HEPES Na, and 0.0001 mM tetrodotoxin, pH 7.4. The intracellular solution contained 100 mM CsF, 10 mM NaCl, 40 mM CsCl, 2 mM $MgCl_2$, and 10 mM HEPES Na, pH 7.2. Exclusion of potassium ions minimized all the components of potassium currents; intracellular fluoride was used to block calcium currents [13]. All the reagents were from Sigma-Aldrich.

The volume of the experimental bath was 200 μl. The external solution was refreshed four times every 3 min by passive flow under gravity. All experiments were performed at room temperature of 22 °C. Ion currents were recorded under the whole-cell patch-clamp configuration [8]. Experiments were controlled by a hardware-software complex consisting of an EPC-7 amplifier, a personal computer, and a self-made software system for the automation of experiments.

The series resistance (R_s) was permanently monitored during all experiments, and maintained at values under 2 MΩ, since this parameter determines the dynamic and stationary errors [18]. Stationary errors in voltage fixation (ΔE_{err}) were estimated as $\Delta E_{err} \cong I_{Na}^{max} \cdot R_s$. When sodium current ($I_{Na}^{max}$) was less than 1 nA, the error did not exceed 2 mV.

Optical scheme. The optical scheme is shown on Fig. 3.1. The radiation source was a continuous-wave CO_2-laser working at the wavelength of 10.6 μm (1).

The radiation power was measured by an IMO-2N laser radiation power meter (Volgograd, Russia) (2) in a beam reflected from the front surface of the optical wedge (3). In order to visualize the position of the CO_2-laser ray, the ray of a semiconductor laser (6) at the wavelength of 0.635 μm was combined with IR ray by means of another optical wedge (5). The radiation power was varied with a disc attenuator (7). The mirror (8) was used to direct the beam into the experimental bath (9) containing the examined neuron. The diameter of the laser beam in the plane of the surface of physiological solution in the bath was 10 mm. Exposure of the neuron to laser radiation was controlled with a mechanical screen (4).

The temperature of physiological solution during irradiation was measured by a calibrated thermocouple Cu-Co, protected from the laser beam by a special screen. The thermocouple was placed in the solution before irradiation to measure the initial temperature. The cross-section distribution of radiation power was assessed in a special experiment by means of calibrated diaphragms positioned in the surface plane of the extracellular solution. The power of radiation that passed through the diaphragm was measured by the IMO-2N power meter placed behind the diaphragm. The hard error of IMO-2N measurements did not exceed 10 %.

In the present experiments the power of radiation in the surface plane of the extracellular solution Ws = 0.23 W and irradiation time was equal to 10 s.

Calculational methods. The full geometry optimization of the ATP^{4-} molecule was performed by the RHF method with 6-31G* basis set [9] within the GAMESS program package [19]. The solvation effects were taken into account in the framework of the PCM model [21]. Using the optimized ATP^{4-} geometry, normal vibrational frequencies were further calculated and the obtained values were scaled with a factor of 0.8929. This method was chosen to adequately simulate the structure of solvated ATP^{4-}, since the vibrational frequencies depend pronouncedly on the set of geometrical parameters.

Statistical analysis. The data were processed with Student's t test at 0.05.

Mathematical modeling of the sodium current kinetics. The gating characteristics of sodium channels were evaluated by mathematical modeling [1, 10]. A basic Hodgkin-Huxley type model of the gating machinery of $Na_v1.8$ channels was constructed earlier [22]. Both the literature (Eq. 3.1) [3] and our experimental data (Eqs. 3.2 and 3.3) were used in the present work to obtain the voltage dependences of the sodium channel rate constants:

$$\alpha_{h\_f} = 0.012\exp(-(E+43)/10) \quad \beta_{h\_f} = 1.32/(1+0.2\exp(-(E+10)/7)] \tag{3.1}$$

$$\alpha_{m\_s} = \exp(0.04E - 0.553) \quad \beta_{m\_s} = \exp(-0.05E - 2.523) \tag{3.2}$$

$$\alpha_{h\_s} = 0.002\exp(-E/30) \quad \beta_{h\_s} = 0.1/[1+0.2\exp(-(E+10)/7)], \tag{3.3}$$

where E is the membrane potential, α_{m_s}, β_{m_s}, α_{h_s}, β_{h_s}, α_{h_f}, β_{h_f} are the rate constants describing voltage dependences of activation and inactivation gating kinetics.

3.3 Results and Discussion

3.3.1 ATP Molecule Is a Probable Target for IR Radiation

ATP molecule is found to have a vibrational absorption band at the wavelength of 10.43 μm with an approximate half-width of 1.11 μm [20], which indicates that this molecule can effectively serve as a receptor of CO_2-laser IR radiation (10.6 μm). As it was earlier shown that Na^+,K^+-ATPase was involved in reception of low-power IR radiation [15], it seems reasonable to assume that Na^+,K^+-ATPase-bound ATP molecules mediate the energy absorption, since fast and undirected dissipation of the absorbed energy into the surrounding medium should be otherwise expected. Most probably, the signal transduction is triggered by ATP molecules bound to the Na^+,K^+-ATPase pumping site on the cytoplasmic side of the neuronal membrane, although a possible involvement of ATP molecules known to participate in phosphorylation of other Na^+,K^+-ATPase loci (*e.g.*, direct phosphorylation by different types of protein kinases) cannot be *a priori* ruled out.

The effective absorption cross-section parameter σ of ATP molecules was measured to be $2.0 \cdot 10^{-17}$ cm^2 [22], which agrees by an order of magnitude with the corresponding cross-section of biological molecules: $\sigma \approx 10^{-16} \div 10^{-17}$ cm^2 [12]. In order to reach the ATP molecule, the radiation has to penetrate across the cellular membrane. The transmittance of a monolayer of spinal ganglia was estimated, and it was found that 11 % of CO_2-laser radiation power could pass through it [22]. Since every ganglion contains a multitude of neuron cells comprising various potential energy absorbers, such as molecules of ATP, water, cell membrane and cytoplasmic organelles, the ability of IR radiation to reach its intracellular receptive target evokes no doubt.

Having thus shown that Na^+,K^+-ATPase-bound ATP molecules are accessible to IR radiation, it is necessary to understand how the radiation energy can be further utilized. Quantumchemical calculations of the normal vibrational frequencies of an ATP molecule were carried out in order to identify the group of atoms that could be responsible for absorption of laser radiation. In the intracellular solution with a physiological pH of 7.2, the ATP molecule assumes a form of a tetraanion ATP^{4-} with a negative charge localized on the triphosphate group. ATP^{4-} vibrational modes appeared to be localized either on the triphosphate group or on the ribose ring, which substantially facilitated the assignment of vibrations.

The calculated vibrational frequencies, their intensities and assignment are presented in Table 3.1. As the experimentally obtained value of ATP vibrational absorption band is 10.43 ± 1.11 μm [20], the data shown are restricted to the range of 9–12 μm. Eight out of fifteen modes are localized on the ribose ring and corresponding hydrogen atoms, the band at 11.39 μm arises from a mixture of three vibrations involving P–O–P and O–P–O bridging bonds, while the other 6 bands are mainly localized on individual phosphate residues constituting the triphosphate group. The closest match to the frequency of laser radiation is the bending vibration of terminal phosphate group (O–P–O)γ at 10.45 μm.

Table 3.1 ATP^{4-} vibrational frequencies in the range of 9–12 μm calculated by RHF method with 6-31 G* basis set within the framework of PCM model, their intensities and band assignment

Wavelength (μm)	Intensity	Assignment
11.83	8.19	Ribose
11.70	13.94	$O_{\alpha\beta}–P_{\beta}–O_{\beta\gamma}$
11.39	10.49	$O_{\alpha\beta}–P_{\beta}–O_{\beta\gamma} + P_{\alpha}–O_{\alpha\beta}–P_{\beta} + O_{\alpha}–P_{\alpha}–O_{\alpha\beta}$
10.66	4.37	$O_{\alpha\beta}–P_{\beta}–O_{\beta\gamma}$
10.49	0.02	Ribose
10.45	9.12	$(O–P–O)_{\gamma}$
10.04	0.13	Ribose
9.83	1.12	Ribose
9.74	2.14	$(O–P–O)_{\beta}$
9.55	6.44	Ribose
9.37	0.99	Ribose
9.34	4.34	$(O–P–O)_{\gamma}$
9.29	1.55	Ribose
9.17	20.06	$(O–P–O)_{\alpha}$
9.13	0.19	Ribose

Thus, the energy of absorbed IR radiation can be localized in the vibrational mode of terminal phosphate residue. Enzymatic ATP hydrolysis is accompanied by the cleavage of terminal $O_{\beta\gamma}–P_{\gamma}$ bond.

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+$$

The radiation energy can be further transferred from the phosphate residue to Na^+,K^+-ATPase by means of the vibrational-vibrational energy exchange, which is a fast process without any energy loss when the two interacting molecules have closely spaced vibrational energy levels. We suggest that the transferred energy results in a change of Na^+,K^+-ATPase conformation, different from the conformational changes characterizing Na^+,K^+-ATPase pumping cycle. This conformational change activates the transducing function of the enzyme, which triggers the subsequent signal transduction to $Na_v1.8$ channels [14, 22].

3.3.2 Slow Sodium $Na_v1.8$ Channel Adequately Senses Low-Power IR Radiation due to Changes in Effective Charge Transfer of Its Activation Gating System

A distinctive feature of $Na_v1.8$ channels is slow kinetics of their inactivation process. This remarkable feature makes it possible to precisely measure the effective charge of the activation gate of this channel (Z_{eff}) using the Almers' method. The tacit assumption of this procedure is the requirement of invariability of

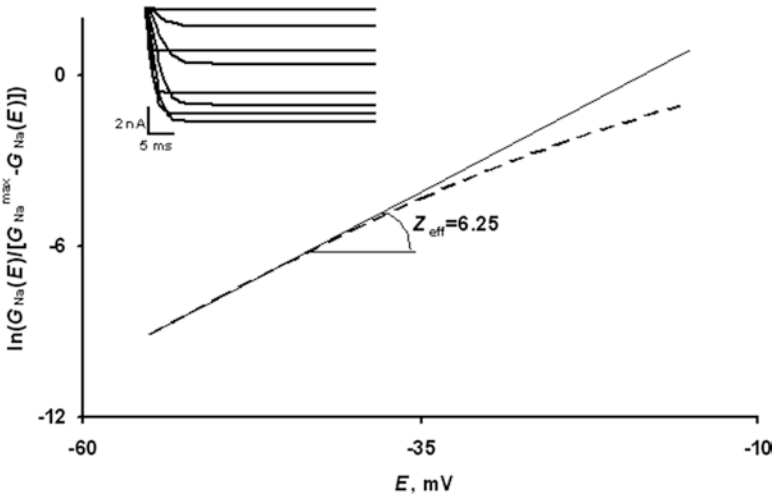

Fig. 3.2 The Almers' procedure for evaluation of the effective charge using the Hodgkin-Huxley theory for the m_s^3-model of $Na_v1.8$ channel activation gating system. The family of sodium currents was calculated using the equation $I(t) = m_s^3(t) \cdot G_{Na}^{max}(E - E_{Na})$ [10] (*inset*), where G_{Na}^{max} is the maximal value of the sodium channel conductance, E_{Na} is the reversal potential for sodium ions. Their amplitude values were used for construction of the Almers' conductance function $G_{Na}(E)/[G_{Na}^{max} - G_{Na}(E)]$ [22]. The voltage dependence of this function in the logarithmic scale is presented as the *dashed line*. The limiting-slope procedure was applied according to the Almers' theory. The slope of the asymptote (*solid line*) makes it possible to determine the value of Z_{eff}

inactivation system behavior at the moment when the value of the peak sodium current is measured [2, 22]. Our calculations show that this assumption is valid only for slow sodium channels, which have very slow inactivation gate kinetics (Eqs. 3.2 and 3.3), and inactivation does not interfere with the activation gating characteristics (Z_{eff}) measurements. The ideal gating model that involves only the activation system of the $Na_v1.8$ channel is presented in Fig. 3.2. Analysis of the effects of slow and fast inactivation system behavior is presented in Figs. 3.3 and 3.4, correspondingly. Figure 3.3 indicates that the slope of the asymptote to the Almers' function passing through the first points determined by the most negative values of the membrane potential gives Z_{eff} equal to 6.19. Taking into account two facts: (1) slow inactivation gating system behavior (Fig. 3.3) influences Z_{eff} negligibly, as compared to the ideal gating model ($Z_{eff} = 6.25$) (Fig. 3.2); (2) the Z_{eff} value derived from experimental measurements of $Na_v1.8$ channel peak sodium currents is in satisfactory accordance with that obtained by the Almers' method [22], it can be concluded that the method can be adequately used for evaluation of Z_{eff} of $Na_v1.8$ channels. The situation changes dramatically when the inactivation kinetics is much faster.

Figure 3.4 illustrates the results of sodium current calculations in a "chimeric" sodium channel. It is supposed that the activation gate of $Na_v1.8$ channel is intact, whereas its inactivation gating system is very fast (as in fast sodium channels). The

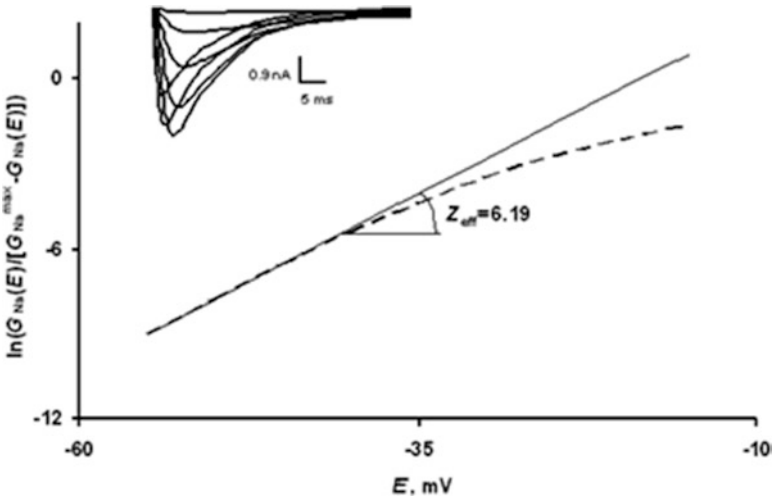

Fig. 3.3 Effective charge calculations using the $m_s^3h_s$-model of $Na_v1.8$ channel gating system. The family of slow sodium currents was calculated using the function $I(t)=m_s^3(t)\cdot h_s(t)\cdot G_{Na}^{max}(E-E_{Na})$ [10] (*inset*). The *dashed line* was obtained as in Fig. 3.2 taking into account the properties of $Na_v1.8$ inactivation system. The slope of the asymptote (*solid line*) makes it possible to determine the value of Z_{eff}

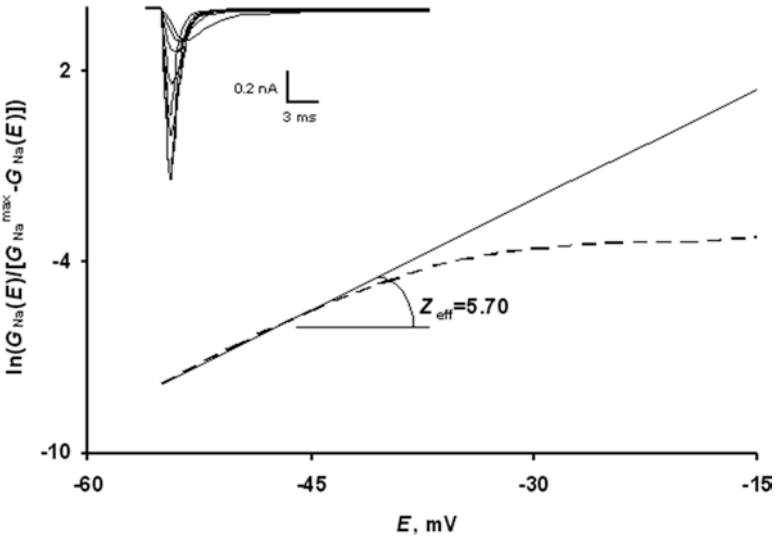

Fig. 3.4 Evaluation of the effective charge for the $m_s^3h_f$-model of a "chimeric" sodium channel with fast inactivation gating system. The family of fast sodium currents was calculated using the function $I(t)=m_s^3(t)\cdot h_f(t)\cdot G_{Na}^{max}(E-E_{Na})$ [10] (*inset*). The *dashed line* was obtained as in Fig. 3.2 taking into account the properties of fast inactivation system. The slope of the asymptote (*solid line*) makes it possible to determine the value of Z_{eff}

Almers' method of Z_{eff} evaluation appears to be noticeably erroneous in this case. The slope of the asymptote passing through the first points gives the value of Z_{eff} equal to 5.70 (Fig. 3.4).

Our recent results show that Z_{eff} is controlled by low-power IR radiation [22]. Application of the very sensitive Almers' method for patch-clamp Z_{eff} measurements makes it possible to obtain the irradiation energy threshold value that induces a Z_{eff} decrease. This energy is approximately equal to the energy of 200 photons emitted by CO_2-laser [16, 22].

The current investigation is also aimed to clarify distinctions between thermal (heating) and non-thermal effects of CO_2-laser IR radiation. Low-power effects of IR radiation should be induced by a molecular mechanism which could not invoke the heating of the surrounding media. Our previous data demonstrate that low-power radiation is sensed by $Na_v1.8$ channel and its activation gating system [22].

3.3.3 *Slow Sodium $Na_v1.8$ Channel Adequately Senses the Threshold of Radiation-Induced Heating due to Changes in Inactivation Gating System*

The combined application of a patch-clamp method and an optical system involving a CO_2-laser [22] was used for the evaluation of the thermal effects of IR radiation. In our experiments, the sodium channel itself was the most effective temperature sensor. The thermal effect, manifested as a temperature elevation upon CO_2-laser irradiation, modulates the kinetics of $Na_v1.8$ channel inactivation gating system. Its thermal response was clearly detected in patch-clamp measurements as a decrease in the duration of the decaying phase of the sodium current trace. The value of this duration (Δt) at the level of the sodium current amplitude equal to 0.5 was considered as the measure of the thermal effect (Fig. 3.5a, b). A statistically significant difference between the control Δt_1 value (without irradiation) and experimental value Δt_2 is presented in Fig. 3.5c. The minimal temperature increase which resulted in a statistically significant decrease in Δt upon irradiation was detected to be 3 °C, from 22 to 25 °C. In our experimental conditions, this temperature threshold corresponds to the energy of ~2,000 emitted by CO_2-laser photons that reached the neuronal membrane.

A more complicated experimental procedure of measurements of inactivation process characteristics yields a similar estimate of thermal energy threshold. The behavior of the inactivation gating system should be more precisely described by the three-state *h*-model [1, 4] (Fig. 3.6).

Assuming that the inactivation gating system has second-order kinetics (Fig. 3.6), the time constants of fast (τ_h) (Fig. 3.7a) and slow (τ_h*) (Fig. 3.7b) exponential processes, as well as the corresponding preexponential factors (Fig. 3.7c), were experimentally obtained. Black squares (Fig. 3.7a, b) indicate that two phases of the inactivation process are significantly accelerated after irradiation in comparison with the control data (white squares). The crosses show that the inactivation process has a tendency to recover after the termination of irradiation.

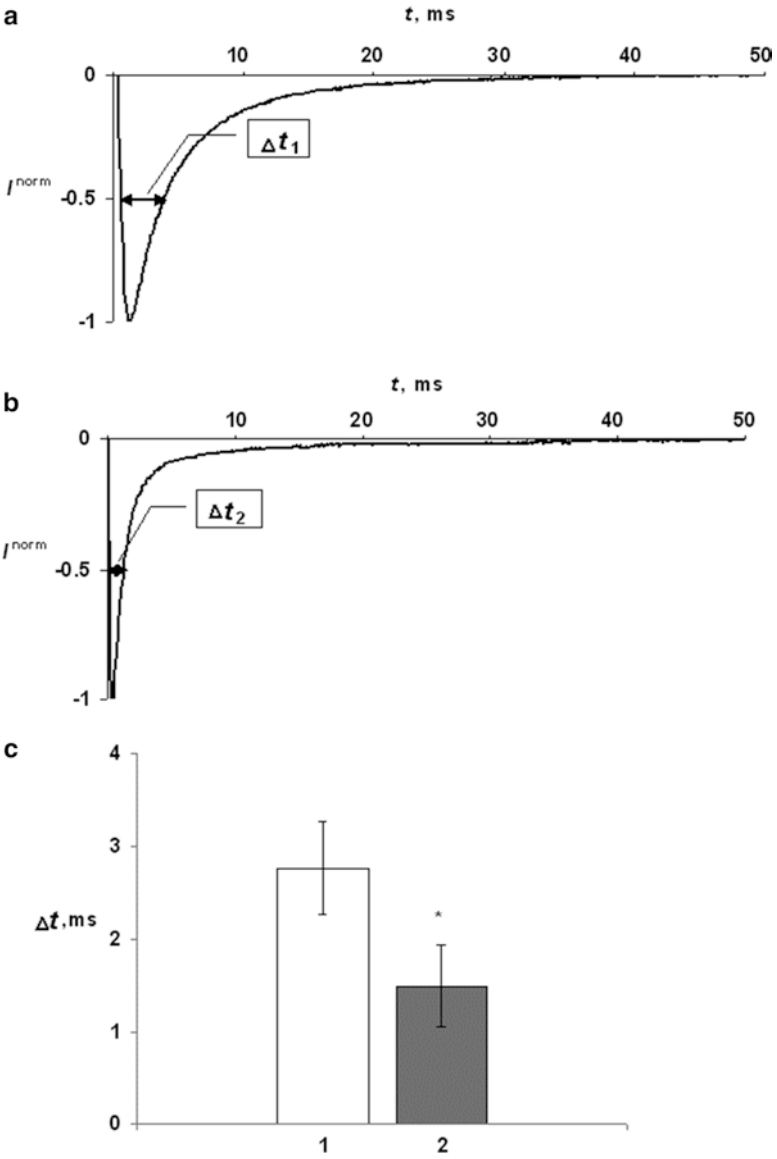

Fig. 3.5 Effects of CO_2-laser irradiation on duration of $Na_v1.8$ channel responses. Duration of the sodium current (Δt) induced by application of a voltage step from −60 to 0 mV to the neuronal membrane was measured at the level of the sodium current amplitude equal to 0.5 in the control experiment (Δt_1, **a**) and immediately after the termination of irradiation (Δt_2, **b**). (**c**) summarizes data on duration of channel responses upon heating of the extracellular solution from 22 to 25 °C: *1* – mean sodium current duration in the control experiments (n = 8), *2* – mean sodium current duration immediately after the termination of irradiation (n = 8). * $p < 0.05$

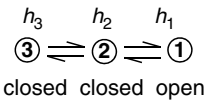

Fig. 3.6 The three-state *h*-model represents the behavior of $Na_v1.8$ channel inactivation gating system

$$h_1(t) = A_1(E) \cdot \exp(-t/\tau_h(E)) + B_1(E) \cdot \exp(-t/\tau_h^*(E))$$

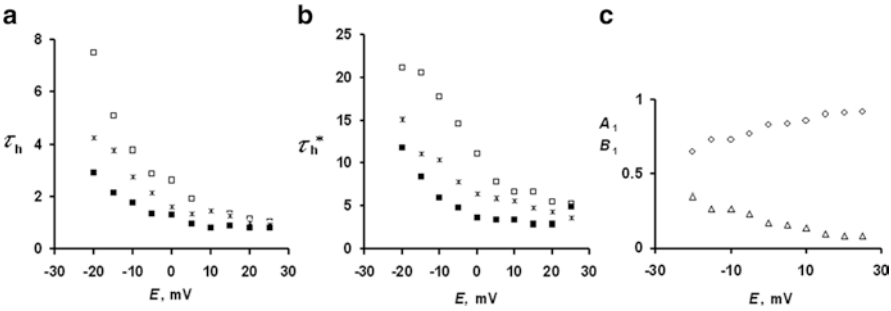

Fig. 3.7 Voltage dependences of τ_h (fast), τ_h* (slow) time constants and pre-exponential factors. (**a**, **b**) Voltage dependences of τ_h and τ_h*, correspondingly, in the control experiments (*open squares*), immediately after the termination of irradiation (*closed squares*), and τ_h recovery 4 min after the termination of irradiation (*crosses*). (**c**) Voltage dependences of fast (*A1*, *open circles*) and slow (*B1*, *open triangles*) pre-exponential factors

A summary of the data showing the effects of IR radiation on τ_h and τ_h* are presented in Fig. 3.8. The elevation of temperature by 3 °C results in a statistically significant decrease in both time constants, which clearly defines the thermal threshold of infrared irradiation. Inactivation system kinetic behavior recovers after the termination of irradiation.

The thermal effects described above occur when the membrane irradiation energy exceeds the energy of ~2,000 photons, and the radiation cannot thus be classified as "low-power". The sensor of this threshold energy is the inactivation gating system of $Na_v1.8$ channels.

The most important conclusion of the present investigation is that low-power CO_2-laser IR radiation invokes a specific response in $Na_v1.8$ channels of sensory neuron membrane. This response is manifested in a relatively narrow threshold) to ~2,000 (upper threshold) photons emitted at the wavelength of 10.6 μm. Only within this range of energies can photons interact with Na^+,K^+-ATPase-bound ATP molecules and by transducer-coupled mechanism [22] they modulate the activation gates of $Na_v1.8$ channels without heating the neuronal membrane. The Z_{eff} of the $Na_v1.8$ channel activation gating system can be adequately measured using the Almers' method range of irradiation energies, which is limited by the energies of ~200 (lower $Na_v1.8$ channels are involved in nociceptive reactions [6]. It is tempting to predict that our *in vitro* observations may be applicable in clinical practice. A decrease

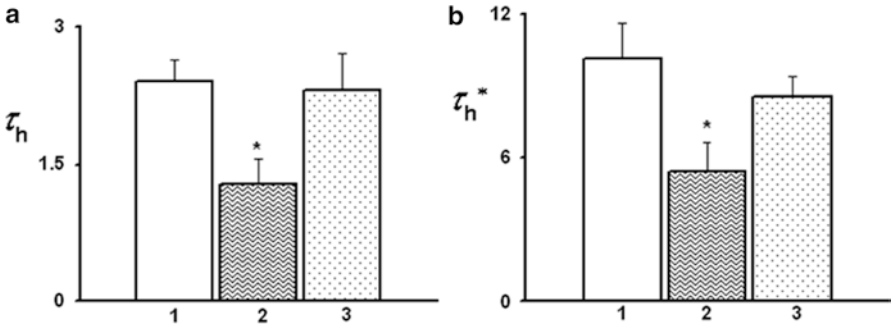

Fig. 3.8 Mean values of τ_h (fast) and τ_h* (slow) time constants. (**a**, **b**) τ_h and τ_h*, correspondingly, in the control experiments (n = 12) (*1*), immediately after the termination of irradiation (n = 12) (*2*), and τ_h recovery 4 min after the termination of irradiation (n = 12) (*3*)

in $Na_v1.8$ channel excitability induced by a decrease in Z_{eff} upon IR irradiation should result in antinociceptive effects, which are expected to be manifested at membrane irradiation energies lying within the range between the energies evoking non-thermal and thermal molecular reactions of nociceptive membrane. A medical device comprising a low-power CO_2-laser, powerful enough to provide the access of IR radiation of the required density to membranes of nociceptive afferents, should effectively reduce the nociceptive signal. Such a device ("Camertone") specifically designed to fulfill the above energetic criteria is currently being clinically tested in the Russian Federation.

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Chapter 4
Correction of the Cancer Therapy-Induced Anemia by the Grape Polyphenol Concentrate Enoant

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Abstract Anticancer drugs do not possess sufficient specificity of action. Each cytostatic agent shows a wide spectrum of side effects that limit the efficacy of therapy and make the quality of life for a cancer patient worse. It forces scientists to search for new pharmacological agents for the correction/prevention of side effects. The polyphenolic compounds are naturally occurring phytochemicals that could be considered as potentially effective protectors of cancer chemotherapy toxicity. The main goal of the study was to study the ability of the grape polyphenol concentrated product Enoant to reduce hematotoxicity of cancer cytotoxic chemotherapy. In a preclinical study, the influence of Enoant on both hematotoxicity and the efficacy of cisplatin administered into Lewis lung carcinoma bearing mice was investigated. Enoant provided a significant protection against cisplatin-induced hematotoxicity by increasing red blood cell production and normalizing leukocyte levels in tumor-bearing mice. Enoant did not stimulate tumor growth and metastasis and did not reduce the efficacy of cisplatin-based therapy. Twenty cancer patients with anemia (averaged hemoglobin level was 85.8±0.8 g/L) were included in the clinical study.

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The correction of anemia during the first stage of investigation was conducted using either iron-containing drugs or erythropoietin. This gave the possibility to begin the course of cancer chemotherapy in 6.7±1.0 days when the hemoglobin level reached 94.6±1.8 g/L. Before the next course of anticancer treatment, the patients received Enoant (total dose of polyphenols was 5 g), that resulted in an 11 % increase of hemoglobin levels ($p<0.05$) up to 103.5±2.8 g/L. Thus, Enoant is an effective and safe agent for the treatment of cancer associated anemia. Enoant is well accepted by patients. Its safety, convenient oral form and palatability make it possible to carry out an effective correction of anemia at home.

4.1 Introduction

Among chronic human diseases, cancer occupies the second place worldwide for mortality (after cardiovascular diseases). In the last century, the major strategy of antitumor therapy was aimed at recovery of cancer patients, i.e. towards complete destruction of the tumor [1, 2]. This strategy also strictly determined the directions of scientific studies in the context of which the anticancer agents with cystatic/cytotoxic action were developed [3, 4]. The main target of antitumor action for these agents was a malignant cell. The main aim was to kill this cell at any cost [5, 6]. However, the beginning of the third millennium was characterized by the formation of a new ideology for which the main goal was a prolongation of life accompanying by an improvement of the quality of life (QOL) of cancer patients [7, 8]. The significant alteration of cancer therapy strategy resulted from low efficacy of cancer therapy against locally advanced and disseminated forms of malignant neoplasms as well as the high toxicity of cancer therapy against normal organs and tissues [9, 10].

It is known that the spectrum of side effects of anticancer therapy is very wide. WHO classifies more than 20 types of side effects of anticancer chemo- and radiotherapy. Hematopoietic injury is one of the most common toxicities of radio- and chemotherapy, resulting in various degrees of neutropenia, anemia or both [11, 12]. Anemia is common in patients with cancer and is a frequent complication of myelosuppressive therapy [13–16]. Approximately one-third of previously untreated cancer patients have anemia, although, it is higher in lymphomas, genitourinary cancers and ovarian cancer where it is up to 50–60 %. Patients receiving myelosuppressive chemotherapy and/or radiation therapy have an even higher incidence of anemia (70–90 %) [11]. It should be noted that certain anticancer agents that are not cytotoxic or myelosuppressive in the traditional sense (imatinib, sunitinib) have nevertheless also contributed to the incidence of anemia [15]. The severity of chemotherapy-induced anemia depends on a variety of factors such as type, schedule and intensity of cancer therapy. Repeated cycles of chemotherapy/radiotherapy may impair erythropoiesis cumulatively [17, 18].

Anemia can decrease a patient's quality of life and leads to delays in treatment and reductions in dose intensity, which compromises the possibility of a favorable outcome and increases the relative mortality risk of cancer patients by about 65 % [13, 19].

However, despite the life-threatening pattern of anemia, an effective therapy may eliminate anemia manifestations and as a result may both improve the quality of life and prolong the lives of cancer patients. The correction of cancer-associated anemia (including radio- and chemotherapy induced) must inevitably improve treatment efficacy and increase the survival of cancer patients [20, 21]. Red blood cell (RBC) transfusions are helpful in the management of anemia in cancer patients, especially when there is a need for immediate increases in hemoglobin levels. The spectrum of pharmacological agents used in clinical practice around the world to treat anemia is not overly large and is presented mainly by erythropoiesis-stimulating agents (ESAs) and iron-containing supplements [22].

A large number of investigations show that the use of ESAs can increase red blood cell production in bone marrow by activating the erythropoietin receptor on erythrocytic-progenitor cells [23]. This results in a significant increase of hemoglobin levels, a decrease of transfusion requirements and an improvement of the quality of life of cancer patients. There are increasing amounts of data from randomized trials displaying better responses to ESAs with the concurrent use of iron-containing supplements. Unfortunately, 35–48 % of these patients show a primary resistance to current treatment with ESAs. In addition, an increased risk of thromboembolic events and a possibly increased risk of mortality and tumor progression (the latter due to the presence of erythropoietin receptors on tumor cells) have been reported in several recent studies [24–27].

The foregoing discussion has, therefore, demonstrated the importance of the search for new pharmacological agents for the management of cancer-associated anemia. Recently, special attention has been focused upon grape polyphenols for some of the pleiotropic health-related beneficial effects (including anti-oxidant, anti-inflammatory, anti-aging, cardioprotective and neuroprotective activities) and toxicity only at extreme doses have been shown [28, 29]. Recent studies revealed an ability of naturally occurring polyphenols to selectively enhance the recovery of lymphocytes and stimulate/protect erythropoiesis [30, 31]. It is well-known that oxidative damage of red blood cells during cancer chemotherapy conditions results in a reduced survival of erythrocytes and a manifestation of anemia. The flavonol quercetin and the stilbenoid resveratrol may show anti-anemic properties due to their ability to inhibit the oxidation of red blood cell membrane proteins and lipids induced by anticancer agents [32]. The protective effects of these polyphenols are enhanced when taken together at low concentrations. The mechanism for the hematopoietic-supportive effect of catechin results from its ability to stimulate the production of granulocyte-macrophage colony forming factor and erythrocyte burst-colony-forming factor [33, 34]. Resveratrol may ameliorate the suppression of erythropoiesis via a modulation of NF-κB signaling in human CD34(+) cells [35]. The results of these investigations support the use of polyphenols (or their combinations) for correction/prevention of cancer associated and/or chemotherapy-induced anemia.

The main goal of the present study was to test the ability of the grape polyphenol concentrate Enoant to correct/prevent anemia induced by cytostatic cancer therapy. Enoant was elaborated from Cabernet Souvignon grapes at the National Institute of

Vine & Wine of the Ukrainian Academy of Agricultural Sciences in 2004. This food concentrate contains high level of grape polyphenols (18–20 g/L of total polyphenols) among which quercetin, catechin and transresveratrol are detected in sufficiently high concentrations. Because any potential modifiers of the toxic action of anticancer drugs may also stimulate tumor growth and metastasis and/or decrease the efficacy of anticancer therapy (both are undesirable actions), this preclinical study of Enoant was carried out prior to clinical investigations.

4.2 Preclinical Study

The investigation was carried out using C57Bl/6 female mice weighing 18–22 g and aged 2–2.5 months. The mice were fed diets with normal protein contents. Mice were bred at the animal facility at the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR). Animal study protocols and operation procedures were approved by the Animal Ethics Committee (AEC).

The cells from the wild-type strain of Lewis lung carcinoma (LLC) were kindly provided by National Bank of Cell Lines and Transplanted Tumors of IEPOR NASU. The cells were cultured *in vitro* in RPMI medium (Sigma, USA) and supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, and 40 μg/mL gentamicin at 37 °C in a humidified atmosphere with 5 % CO_2.

After intramuscular inoculation of tumor cells (1×10^6 cells per animal in 0.1 mL of Hanks' solution), all animals were randomized by weight and assigned to groups (10 animals per group). Mice of two experimental groups were treated by Cisplatin in combination with either Enoant or Quercetin (group "CP+E" and group "CP+Q", correspondingly). The animals treated with Enoant (group "E"), Quercetin (group "Q") or Cisplatin (group "CP") were used as positive controls. Mice in the negative control groups received water (group "C") by schedule and volume similar to that for groups treated with the test agents.

Enoant (2.5 mL/kg of body weight per day) was administered per os by orogastric gavage technique daily for 2 weeks, starting from the ninth day after tumor cell inoculation. Quercetin (50 mg/kg of body weight per day) was administrated intraperitoneally three times a week during 3 weeks, starting from the second day after tumor cell inoculation. Cisplatin (0.8 mg/kg of body weight per day) was administered intraperitoneally every other day, starting from the 11th day after cancer cell inoculation.

Mice from all groups were sacrificed on the 23rd day after tumor cell inoculation. The volume of the primary tumor as well as the total volume of the lung metastases and the number of metastatic nodules was estimated. Sampling of bone marrow from the mouse femur was carried out and the cellular content of the marrow tissue was analyzed.

The statistical analysis of the results included descriptive statistics, Student's *t*-test, and Mann–Whitney *U*-test and was performed using Statistica v.8.5.

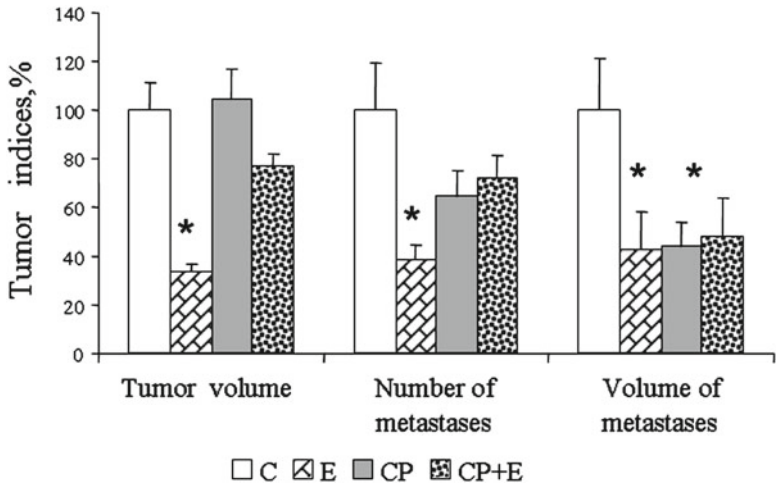

Fig. 4.1 The effect of Enoant on LLC growth and metastasis. LLC-bearing mice were treated with Enoant (group "E"), Cisplatin (group "CP"), and Enoant in combination with Cisplatin (group "CP+E"). Negative control group is marked as "C". *$p < 0.05$ vs. control

4.2.1 Results and Discussion

The results obtained in our study showed that Enoant in monotherapy regime did not stimulate tumor growth and metastasis and in combination with Cisplatin did not reduce the efficacy of the anticancer drug (Fig. 4.1). Moreover, therapy with Enoant alone resulted in a statistically significant decrease of tumor volume, the number of metastatic nodules and the total volume of lung metastases by approximately 60 % ($p < 0.05$) as compared to the negative control group.

The low anticancer efficacy of Cisplatin in the study can be explained by the relatively high resistance of Lewis lung carcinoma to a wide spectrum of anticancer drugs, as well as the relatively low dose of Cisplatin. The combined therapy of Cisplatin with Enoant showed a trend for tumor growth inhibition, which did not reach statistical significance due to the high variability of the parameters studied. This trend may in part be explained by the action of Quercetin, one of the components of Enoant. It has been shown that co-administration of Quercetin and Cisplatin can inhibit tumor growth by approximately 58 % ($p < 0.05$), and decrease the metastatic volume by 78 % ($p < 0.01$) compared with the negative control group. At the same time, monotherapy with Quercetin did not affect LLC growth and metastatic activity.

The results of the investigations on the cellular composition of bone marrow (playing a major role in hematopoiesis and being a target of cytotoxic action of cancer chemotherapy) are presented in Fig. 4.2. An analysis of bone marrow cytograms of LLC-bearing mice after treatment with Cisplatin displayed an abnormality

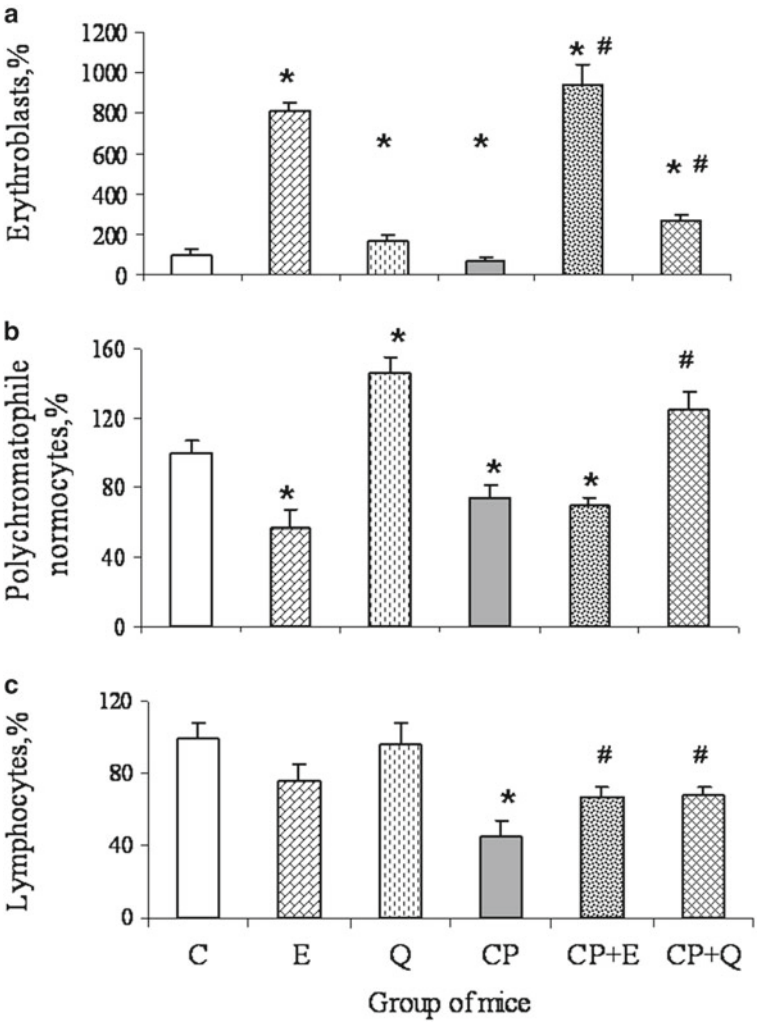

Fig. 4.2 The effect of Enoant and Quercetin on the hematopoietic system: (**a**) the number of erythroblasts, (**b**) the number of polychromatophile normocytes, and (**c**) the number of lymphocytes in bone marrow of LLC-bearing mice. LLC-bearing mice were treated with Enoant (group "E"), Quercetin (group "Q"), Cisplatin (group "CP"), and Enoant or Quercetin in combination with Cisplatin (group "CP+E" and "CP+Q" correspondently). Negative control group is marked as "C". *, #$p < 0.05$ with respect to control and group "CP", respectively

in all branches of hematopoiesis in bone marrow. This was particularly evident in the suppression of erythrocyte cell proliferation and a reduction of the lymphocyte count.

A significant suppression (>30 %, $p < 0.05$) of erythropoiesis in a group of mice treated with Cisplatin confirms previous findings that this cancer drug can inhibit the proliferation and differentiation of erythrocytes.

Cytomorphologic analysis of bone marrow myelograms of mice after treatment with Enoant alone or in combination with Cisplatin revealed a significant ($p < 0.001$) and powerful stimulation of erythrocytes (Fig. 4.2a). This stimulation was manifested in an eight- to ninefold increase in the number of erythroblasts in the bone marrow of treated mice in comparison with that of the control group. Despite the pronounced ability of Enoant to increase the erythroblast content, the number of polychromatophile normocytes after administration of Enoant alone or in combination with Cisplatin remained lower than that of the control group (Fig. 4.2b). The ability of Enoant to stimulate the erythroid branch of hematopoiesis may have been caused in part by Quercetin. It was shown that treatment of tumor-bearing mice with Quercetin alone or in combination with Cisplatin resulted in a statistically significant two- and fourfold increase, respectively, in the number of erythroblasts compared to a negative control. In addition, Quercetin did not reduce the number of polychromatophile normocytes. This effect was not observed with Enoant.

In addition to the inhibition of the erythroid line of hematopoiesis, Cisplatin caused a statistically significant ($p < 0.001$) twofold decrease of lymphocyte number in bone marrow of LLC-bearing mice (Fig. 4.2c). When Cisplatin was administered in combination with Enoant or Quercetin, a normalization of lymphocyte levels was observed. It was confirmed by a 1.5-fold ($p < 0.05$) increase in lymphocyte number in bone marrow of mice from "CP+E" and "CP+Q" groups, compared to that of mice treated with Cisplatin alone.

Thus, the results from our preclinical study have revealed a significant protective effect of the grape polyphenol concentrate Enoant against Cisplatin-induced hematotoxicity by increasing a red blood cell production and normalizing leukocyte levels. Enoant did not stimulate tumor growth and metastasis. In contrast, Enoant showed an antitumor activity against Lewis lung carcinoma. As the most significant protective effect of Enoant was with respect to erythropoiesis, the main goal of the clinical study was to prove the capacity of Enoant to correct/prevent cancer and/or chemotherapy-induced anemia.

4.3 Clinical Study

Twenty cancer patients with anemia (hemoglobin level <90 g/L estimated at the moment of hospitalization) were included in the study. Hospitalization was caused by the cancer chemotherapy (CT). The patients (80 % females, 20 % males) were diagnosed with breast cancer (35 % cases), oncogynecological cancer (30 % cases), lymphoma (15 % cases), and single cases of lung cancer, rectal, thyroid and testicular cancers. The median age was 55.4 years (range: 32–74). Thirty percent of the patients were successfully treated in the hospital for the first time, and respectively, their anemia was caused by tumor development.

In 70 % of the cases, the disease duration was on average 29 ± 6 months. Anemia in these patients resulted from both tumor development and cancer therapy. Ninety-three percent of the patients received 6.5 ± 1.3 courses of chemotherapy mainly with

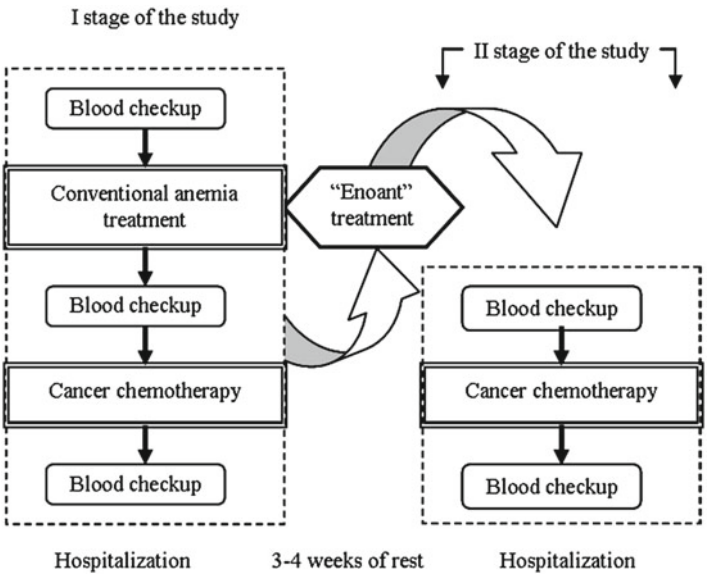

Fig. 4.3 Clinical study design

anthracycline antibiotics and platinum-containing drugs. Fifty percent of the patients underwent radiotherapy.

The structure of the clinical studies is presented on Fig. 4.3. During the first hospitalization, all patients were assigned to receive conventional anemia treatment that included iron-containing drugs Venofer (80 % patients) and recombinant beta erythropoietin Recormon (20 % patients). During CT, all patients were recommended to receive a protein-rich diet.

At the first and second hospitalizations, CT was initiated only when the hemoglobin level was higher than 90 g/L. In the period between the first and second hospitalizations (the duration of which was determined by CT regimes and was 21–28 days), the patients received 15 mL of Enoant per os daily. This was divided into three doses for 17 consecutive days starting on the foruth day after the completion of one course of anticancer therapy. The total dose of Enoant was 250 mL.

The clinical status of the cancer patients, blood tests and biochemical analyses of blood (including analysis of coagulogram) was carried out when the patient was first admitted to the hospital, prior to CT and at the fourth day after CT completion.

4.3.1 Results and Discussion

According to international classifications, a hemoglobin level of 90 g/L is an indication of moderately expressed anemia [36]. In oncological practice, this level is identified as a threshold to allow cancer patients to undergo cytotoxic therapy.

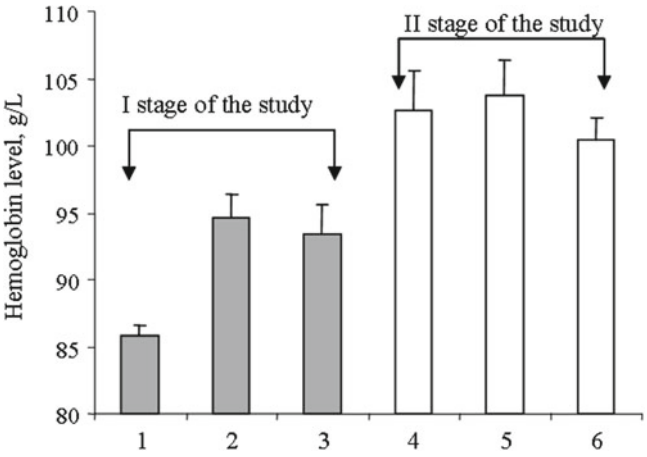

Fig. 4.4 The changes of hemoglobin level in patients during clinical study: *1* and *4* – time of hospitalization; *2* and *5* – day before cancer chemotherapy; *3* and *6* – fourth day after cancer therapy completion

At the moment of the first (in the frame of the study) hospitalization, the hemoglobin level of all patients was lower than this threshold and on average equaled 85.8 ± 0.8 g/L (mean ± s.e.m) (Fig. 4.4). Conventional anemia treatment resulted in an increase of hemoglobin content in 85 % of patients. Thus, prior to the first course of CT, the average hemoglobin level was 94.6 ± 1.8 g/L. Correction of anemia for these patients caused about a 7-day delay of CT (Table 4.1). It is necessary to note that conventional therapy against anemia did not induce an elevation of hemoglobin levels to up to 90 g/L for three patients stipulating the necessity of cancer drug dose reduction for two of them and cancellation of anticancer therapy for the third patient.

At the moment of second hospitalization after Enoant treatment, the level of hemoglobin in 90 % of the patients studied was higher than 90 g/L. At the beginning of the second course of CT, the average hemoglobin level was 104.5 ± 2.5 g/L. This was 10 % ($p<0.001$) above this index prior to CT during stage I of the study. This allowed the physicians to perform CT without any delay and without a reduction in dosage. This resulted in more effective protocols of cancer chemotherapy.

The hemoglobin content in the blood from two patients at the second hospitalization was lower than 90 g/L, which caused a 3-day delay in CT. During this period, these patients did not receive any anemia treatment, so one may suppose that increase of their hemoglobin level was caused by Enoant.

Administration of Enoant did not significantly influence leukocyte or platelet counts (Table 4.1). Moreover, a tendency towards their stabilization was fixed. Analysis of biochemical blood indices and clinical status of the patients did not reveal any side effects of Enoant (Table 4.2).

In addition to the significant anemia, most of the patients at the beginning of the study demonstrated considerable abnormalities in the blood clotting system. This was manifested as an increase in the fibrinogen level of approximately 30 % above

Table 4.1 Changes of hematological indices of cancer patients during the first and second stages of the clinical study

	Hematological indices		
Time of blood checkup	HB (g/L)	WBC (10^9/L)	PLT (g/L)
I stage of the clinical study			
Time of hospitalization	85.8 ± 0.8	7.5 ± 1.2	329.0 ± 44.0
Prior cancer therapy	94.6 ± 1.8	7.7 ± 1.3	310.0 ± 32.0
Fourth day after cancer therapy completion	93.6 ± 2.4	6.4 ± 0.6	258.0 ± 41.0
II stage of the clinical study			
Time of hospitalization	102.6 ± 3.0	6.9 ± 0.7	286.0 ± 31.0
Prior cancer therapy	103.5 ± 2.8	7.1 ± 0.6	281.0 ± 30.0
Fourth day after cancer therapy completion	100.4 ± 1.8	6.4 ± 0.7	250.0 ± 29.0

Note: *HB* hemoglobin content, *WBC* white blood cells, *PLT* platelet number. Results are represented as means and corresponding standard errors

Table 4.2 Coagulogram and biochemical indices of cancer patients before CT of the first and second stages of the clinical study

	Coagulogram indices		Blood biochemistry		
Stage of the clinical study	Fibrinogen (g/L)	Prothrombin index (%)	Protein (g/L)	Creatinine (μmol/L)	Bilirubin (μmol/L)
I	5.4 ± 0.5	86.1 ± 1.7	70.5 ± 1.6	90.9 ± 3.1	7.9 ± 0.2
II	4.6 ± 0.4	84.5 ± 1.6	72.4 ± 1.6	87.4 ± 2.9	8.6 ± 0.9

Note: Results are represented as means and corresponding standard errors

the upper limit of the norm (Table 4.2). Such changes of the blood clotting system are typical for cancer. After Enoant therapy (at the beginning of the second stage of the study), the fibrinogen level was reduced by 15 % ($p < 0.05$). Normalization of this index may reflect the anticancer efficacy of the cancer chemotherapy, and/or it may be caused by the action of Enoant.

4.4 Conclusion

The results of the studies performed here have shown that Enoant is an effective and safe agent for the treatment of cancer associated and chemotherapy-induced anemia. Enoant is well accepted by patients. Its safety, convenient oral form and palatability support its use as an effective means to correct anemia at home.

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Chapter 5
Radioprotective Properties of Selenomethionine with Methionine, Extracts from *Basidium* Fungi and Exogenous DNA

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Abstract The search for effective substances to provide protection to the organism under radiation treatment continues to be one of the most important directions for studies in radiobiology. The results of the study show that selenmethionine in the indicated doses with methionine (4 mg/kg) have pronounced efficiency on the defense of organisms against acute irradiation at a dose of 2 Gy. This was shown in the recovery of leucocytes in the blood, the increase in the total number of spermatogenic cells, and a significant increase in the spermatid count by 2 months after irradiation (2.3–2.6 times). This may provide a restoration in the process of spermatogenesis as well as normalization of lipid peroxidation processes and transamination enzymes activities in the irradiated animals. We also studied the anti-tumoral and radioprotective activities of the mushroom aqueous extracts. The antitumoral properties of *Flammulina velutipes* are shown. Its aqueous extracts decrease a spontaneous level of adenomas and, as well as *Auricularia auricular-judae*, reduce the yield of urethane-induced adenomas. The aqueous extracts of *Flammulina velutipes* and *Phallus impudicus* demonstrate radioprotective properties, i.e. reduce a number of adenomas in irradiated mice, raise mice survival and stimulate a growth of endogenous colonies in spleen. We prepared and studied the exogenous DNA. Micronuclei tests of bone marrow show an improvement in the DNA repair process in erythroblasts of irradiated and treated animals. Based on PCR analysis, it was suggested that the exogenous DNA substance can protect cardiac cells from the activation of pro-apoptotic gene Bax, and it can rescue the heart from irradiation-induced apoptosis. Thus the studied substances can be promising agents for protection and recovery of an organism from radiation injury.

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5.1 Radioprotective Effects of Selenmethionine with Methionine

5.1.1 Introduction

The search for effective substances to provide protection to an organism under radiation treatment continues to be one of the most important directions of studies in radiobiology [1]. In this regard, microelement selenium and amino acid methionine are of considerable interest. Selenium is part of the antioxidant enzyme glutathione peroxidase, which catalyzes hydrogen peroxide and the reduction of lipid hydroperoxides and protects cell membranes from the damaging action of lipid peroxidation products [2], which accumulate in the tissues of the body under irradiation. Methionine is an essential amino acid, which plays an important role in the methylation and synthesis of amino acids and proteins [3]. However, the use of selenium in the form of inorganic salts or organic compounds has serious constraints because they are poorly metabolized. Selenium replacement of the sulphur atom in the selenmethionine molecule resulted in the occurrence of one of the most assimilated forms of organically bound selenium that open the way for its wider use. The purpose of this work was to assess the radioprotective properties of selenmethionine with methionine under exposure to γ-rays in acute dose of 2 Gy.

5.1.2 Materials and Methods

Experiments were performed on white male rats. Acute radiation exposure of animals (age 3.5 months, weight 331 ± 15 g) was made at IGUR-1 (^{137}Cs, dose rate 0.92 Gy/min) at a dose of 2 Gy. The animals were divided into five groups: (1) untreated control; (2) animals exposed to irradiation in a dose of 2 Gy; (3) animals exposed to irradiation in a dose of 2 Gy and receiving selenmethionine in a dose of 1.5 μg/kg + 4 mg/kg of methionine; (4) animals exposed to a dose of 2 Gy and receiving selenmethionine in a dose 15 μg/kg + 4 mg/kg methionine; (5) animals exposed to irradiation in a dose of 2 Gy and receiving selenmethionine in dose 150 μg/kg + 4 mg/kg of methionine. The mixture of substances in these doses was injected for 3 days before and 5 days after the irradiation. In the later case the substances were injected twice a week for 1 month after exposure. Experiments were made at the 7th, 30th and 60th day after irradiation. Blood of animals was sampled, smears were prepared and serum was received; testes, epididymise and liver tissues were removed. The number of leucocytes in the blood and leukogram were determined. The number of all types of spermatogenic cells and it total quantity [4] as well as the number of spermatocytes isolated from epididymeses [5] were calculated. In serum, liver and testis tissue homogenaties the content of malonic dialdehyde (MDA) [6] and the total antioxidant activity (TOA) [7] were determined. The obtained data were generally accepted by variational statistics methods using Student's *t*-test at significance level $P < 0.05$.

Table 5.1 The effects of selenmethionine with methionine on the leukocytes quantity and blood leukogram of rats after an acute irradiation in dose of 2 Gy

	Series of experiments				
Studied indices, 10^9/l	Control	2 Gy	2 Gy + P_1	2 Gy + P_2	2 Gy + P_3
7th day after exposure					
Leucocytes	7.1 ± 0.6	2.9 ± 0.2*	4.2 ± 0.5*	4.8 ± 0.6	5.4 ± 0.4
Eosinophils	0.10 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
Monocytes	0.07 ± 0.03	0.04 ± 0.02	0.05 ± 0.02	0.07 ± 0.02	0.11 ± 0.20
Neutrophils	0.96 ± 0.12	0.93 ± 0.07	1.38 ± 0.04*	1.44 ± 0.07*	1.54 ± 0.07*
Lymphocytes	5.98 ± 0.11	1.91 ± 0.08*	2.76 ± 0.05*	3.26 ± 0.05*	3.71 ± 0.07*
30th day after exposure					
Leucocytes	5.36 ± 0.45	4.88 ± 0.66	5.80 ± 0.99	3.70 ± 0.34*	5.04 ± 0.59
Eosinophils	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
Monocytes	0.10 ± 0.02	0.16 ± 0.01	0.10 ± 0.00	0.13 ± 0.03	0.12 ± 0.02
Neutrophils	0.55 ± 0.01	1.00 ± 0.01*	0.71 ± 0.01	0.54 ± 0.03	0.79 ± 0.08
Lymphocytes	4.69 ± 0.06	3.67 ± 0.08*	4.93 ± 0.14	3.03 ± 0.06*	4.11 ± 0.09*

Note: *P1* – selenmethionine (1.5 μg/kg) + methionine (4 mg/kg); *P2* – selenmethionine (15 μg/kg) + methionine (4 mg/kg); *P3* – selenmethionine (150 μg/kg) + methionine (4 mg/kg); *P < 0.05 vs. control

5.1.3 Results

On the seventh day after acute exposure at a dose of 2 Gy there was a sharply pronounced leukopenia in the blood of animals: the leukocytes count was decreased compared to the control of almost 2.5-fold (Table 5.1). Substantial changes were also detected in the leukogram of irradiated animals. Administration of selenmethionine in doses of 1.5, 15 and 150 μg/kg in combination with methionine (4 mg/kg) has a positive effect on blood leukocytes of irradiated animals (the number increased by 1.45–1.86 fold).

By the 30-th day after irradiation, the quantity of leukocytes in the blood increased, but did not reach the control level. Selenmethionine in minimum (1.5 μg/kg) and maximum (150 μg/kg) concentrations with methionine (4 mg/kg) led to a normalization of the number of leukocytes in the blood of rats subjected to irradiation. The leukogram of irradiated animals and animals treated with these substances on the background of irradiation was characterized by a restoration of cellular composition of individual elements of the system of blood leukocytes. By the end of the study (60th day), in the blood of irradiated animals, a significant leukopenia (4.3 ± 0.6 % as compared to 7.2 ± 0.4 % in control) remained. The irradiated animals that received selenmethionine with methionine were characterized by low levels of leukocytes in the blood, probably due to the termination of the effects of substances which were delivered only till the end of the first month after exposure. Thus, the action of selenmethionine in doses of 1.5; 15 and 150 μg/kg with methionine (4 mg/kg) on the restoration of the blood leukocytic system of the irradiated animals was effective as evidenced by the number of leukocytes and other leukocytic elements. On the seventh day after exposure, the number of different types of germ cells did

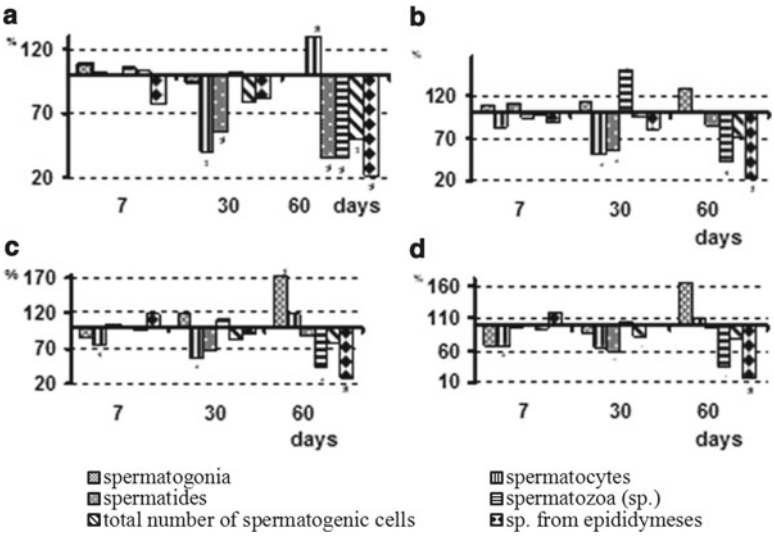

Fig. 5.1 The effect of selenmethionine with methionine on the content of spermatogenic cells in rat testes and epididymis after acute irradiation at a dose of 2 Gy. A – irradiation 2 Gy; B – irradiation 2 Gy + selenmethionine 1.5 mkg/kg + methionine (4 mg/kg); C – irradiation 2 Gy + selenmethionine 15 mkg/kg + methionine (4 mg/kg); D – irradiation 2 Gy + selenmethionine 150 mkg/kg + methionine (4 mg/kg). *$P < 0.05$ vs. control

not differ from the control (Fig. 5.1). However, in the subsequent period, the quantity of separate types of spermatogenic cells sharply decreased. After 1 month after exposure, the counts of spermatocytes and spermatids dropped to 60.5 and 44.5 %, respectively, and after 2 months the quantity of testicular spermatids and spermatozoa fell by almost threefold. The number of spermatozoa isolated from epididymeses was greatly reduced on the 7th and 30th day (to 23 and 19 %, respectively) after irradiation and declined by 2.7 fold after 2 months.

It was estimated that on the seventh day after acute irradiation there were no noticeable changes in the quantity of spermatogenic cells in the testes under the influence of selenmethionine with methionine. However, an acceleration of the entrance of mature sex cells in the epididymis was observed after administration of the substances was revealed, accompanied by an increase in their numbers. The effects of selenmethionine with methionine on the content of spermatogenic cells in the testes of rats after 1 month of radiation exposure at a dose of 2 Gy resulted in an increase of individual forms of sex cells, which also resulted in a marked increase in their total number This was especially expressed at a minimum concentration of selenmethionine (1.5 μg/kg) with methionine (4 mg/kg). At the same time in these conditions (radiation exposure at a dose of 2 Gy + experimental substances), there were no reliable changes in the number of spermatozoa isolated from the epididymis.

Two months after an acute bout of irradiation, selenmethionine with methionine had a pronounced effect on the number of spermatogenic cells in the testes of

Table 5.2 The effects of selenmethionine with methionine on selected indices of antioxidants in rat tissues after different periods of acute γ-irradiation at a dose of 2 Gy

	Series of experiments				
Studied indices	Control	2 Gy	2 Gy + P_1	2 Gy + P_2	2 G + P_3
7th day					
MDA (b), nmol/l	25.4 ± 1.8	41.4 ± 1.4*	31.5 ± 1.2$^{\#}$	26.4 ± 0.9$^{\#}$	18.8 ± 1.5$^{\#}$
MDA (l), nmol/g	2.22 ± 0.09	4.19 ± 0.12*	2.78 ± 0.09$^{\#}$	2.80 ± 0.09$^{\#}$	2.18 ± 0.06$^{\#}$
TOA (l), relative units	0.80 ± 0.12	0.50 ± 0.02*	1.03 ± 0.36	1.23 ± 0.08$^{\#}$	1.14 ± 0.08$^{\#}$
30th day					
MDA (b), nmol/l	30.2 ± 1.9	43.5 ± 5.5	20.2 ± 0.8$^{\#}$	26.1 ± 3.2$^{\#}$	32.6 ± 2.1
MDA (l), nmol/g	2.87 ± 0.21	3.94 ± 0.15*	3.23 ± 0.20	2.83 ± 0.20$^{\#}$	2.75 ± 0.15$^{\#}$
TOA (l), relative units	1.40 ± 0.30	0.75 ± 0.08	1.03 ± 0.30	1.00 ± 0.09	0.95 ± 0.35
60th day					
MDA (b), nmol/l	15.3 ± 1.7	31.4 ± 3.8*	21.6 ± 3.0	16.1 ± 1.3$^{\#}$	18.4 ± 1.1$^{\#}$
MDA (l), nmol/g	3.24 ± 0.18	3.56 ± 0.13	2.93 ± 0.08$^{\#}$	2.75 ± 0.10$^{\#}$	2.64 ± 0.03$^{\#}$
TOA (l), relative units	1.20 ± 0.08	0.80 ± 0.33	1.05 ± 0.35	1.18 ± 0.09	1.15 ± 0.06

Notes: (b) – blood; (l) – liver; *$P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. irradiation

rats at the stage of spermatids. The count was increased in 2.3–2.6 times compared with radiation. As a result, there was a significant increase in the total number of spermatogenic cells. However, the number of sperm isolated from epididymis, when under the influence of substances in different doses, did not only promote their restoration, but reduced the quantity of these cells compared with those in the exposed animals. Lowering the number of sperm in the epididymis of the irradiated animals and irradiated animals after the administration of selenmethionine with methionine was due in part that for this period the effect of radiation on cells was at its peak. This is likely because the cycle of the spermatogenic epithelium in the rat was 50 days. However, given the significant enhancement of the spermatid count in the testes in that period, it should be assumed that a normalization of the process of spermatogenesis in the spermatogennic epithelium occurs as the germ cells mature in the epididymis in animals fed the experimental substances. Therefore, the introduction of selenmethionine with methionine to animals, once irradiated in dose of 2 Gy, promoted restoration of spermatogenous cells in the testes.

Table 5.2 presents data on the action of acute irradiation at a dose of 2 Gy on the MDA content in various tissues of male rats with and without the influence of selenmethionine with methionine. On the seventh day after exposure, it was estimated that the MDA level increased to 163 % in the serum, and increased to 189 % in liver as compared to the intact control. In accordance with the increase of the content of MDA in the liver tissue of irradiated animals, a reduction in the antioxidant activity (by 37.5 %) took place. Administration of selenmethionine to rats subjected to acute irradiation in a dose of 2 Gy led to a decrease in the concentration of MDA in the serum and liver. This was more significant at a maximum dose of selenmethionine (150 μg/kg) with methionine (4 mg/kg). In this case, the content of MDA in the tissues of irradiated animals decreased below the reference

Table 5.3 The content of MDA in rat testis tissue in different period after acute irradiation at a dose of 2 Gy and with administration of selenmethionine with methionine

	Post-irradiation period, day		
Series of experiments	7th	30th	60th
Control	0.205 ± 0.090	0.146 ± 0.060	0.216 ± 0.090
2 Gy	0.164 ± 0.070*	0.222 ± 0.080*	0.277 ± 0.100
2 Gy + P_1	0.200 ± 0.080	0.117 ± 0.050*	0.237 ±0.140
2 Gy + P_2	0.239 ± 0.080*	0.179 ± 0.070*	0.221 ± 0.120
2 Gy + P_3	0.256 ± 0.070*	0.189 ± 0.070*	0.473 ± 0.150

Notes: the designations are the same as in Table 5.1

level, which supported the effective action of selenmethionine with methionine at the level of lipid peroxidation. Simultaneously with the fall in the content of MDA in the liver of animals subjected to external irradiation at a dose of 2 Gy and receiving the drug, an increase in antioxidant activity was observed. In these conditions, the reaction of the spermatogenic epithelium tissue at the radiation impact (Table 5.3) had a different nature in comparison with that in the serum and liver. On the seventh day after exposure, the reduction of MDA content in the testes tissues was 20 %, and the drug slightly increased this parameter in the presence of 1.5 μg/kg concentration of selenmethionine with methionine (4 mg/kg) and enhanced it more significantly by increasing the selenmethionine concentration up to 15 μg/kg. On the 30th day, the content of MDA in tissues of irradiated animals, including the spermatogenic epithelium, was significantly higher. In all experiments using selenmethionine in combination with methionine, we observed a normalizing effect on the reduction of the concentration of the final product of lipid peroxidation – MDA in irradiated animals.

Changes in TOA indices in rat liver corresponded to MDA levels. The direction of the changes in lipid peroxidation in the serum, liver and testicular tissue of animals after the 60th day of irradiation and the administration of selenmethionine with methionine was similar. This was typical for the previous series, with the exception of the experiments where higher concentrations of selenmethionine were tested on the levels of MDA in the testicular tissue. In general, there was a positive effect of the substances in most series of experiments on the normalization of lipid peroxidation state in animal tissues after acute irradiation at a dose of 2 Gy.

The results of the study demonsrate that selenmethionine in the indicated doses and combined with methionine (4 mg/kg) can have a pronounced effect on the defense of organisms against acute irradiation at a dose of 2 Gy. This was evident from the restoration of the quantity of leukocytes and leukocytic blood elements, an increase in the total number of spermatogenic cells, due to the growth of different types of germ cells at different stages of differentiation, and a significantly increased spermatid count after 2 months of irradiation (2.3–2.6 times). This seems to provide a restoration in the future of the process of spermatogenesis as well as a normalization of lipid peroxidation processes in the irradiated animals.

5.2 Antitumor and Radioprotective Effects of Extracts from *Basidium* Fungi

5.2.1 Introduction

The properties of *Basidiomycetes* and their practical employment in medicine are being actively investigated by scientists of many countries including China, Japan, Korea and the USA. Biologically active substances from higher *Basidiomycetes* possess antibacterial, antiviral and antifungal properties, pronounced radiatioprotective effects, demonstrate anti-inflammatory, hypocholesterolemic and hepatoprotective effects [8]. Historically, in Belarus, the fruit-bodies of *Phallus impudicus* have long been popular amongst mushrooms and an important factor in folk medicine for the treatment of stomach ulcers, skin injuries and all local tumors. In recent years, an interest in *Basidiomycetes* as new source of efficient and safe antitumor activity has been cultivated [9]. The antitumoral activity of the substances and preparations from mushrooms has been shown to suppress angiogenesis, and to induce apoptosis in a cancerous cell culture via activation of proapoptotic proteins and caspases via mitochondrial cytochrome C [10]. It has also been shown to reduce the activity of B-cells and to potentiate activation of T-helpers [11], to increase the number of monocytes and granulocytes in mice during leukopenia induced by the injection of cyclophosphan [12]. Therefore, the objectives were to estimate the influence of aqueous extracts from selected mushroom species on the growth and development of tumor process in lungs of *Af* mice as well as to explore their radioprotective activity.

5.2.2 Materials and Methods

The antitumoral activity of the mushroom aqueous extracts *Flammulina velutipes* and *Auricularia auricular-judae* (grown in the laboratory of food and medicinal resources of forest (Forest Institute of NAS of Belarus)) and *Phallus impudicus* (gathered in the forests of the Gomel region) was investigated. The antitumoral activity of aqueous extracts of mushrooms was assessed in response to spontaneous and induced (with the chemical mutagen urethane and radiation exposure) lung adenomas in *Af* mice. The typical features of *Af* mice are the hereditary propensity for carcinogenesis in lungs and the high sensitivity of lung tissue to the blastomogenic action of urethane. The number of spontaneous lung tumors in intact mice increases after exposure to mutagenic and carcinogenic factors.

The experimental animals (male and female) were separated into six groups: 1 group – intact mice were given a single introperitoneal (i.p.) injection of physiologic saline (10 ml/kg); 2 group – intact mice were given a single injection (i.p.) of urethane (1 mg/kg); 3 group – mice were allowed to drink an aqueous extract of *Auricularia auricular-judae* (4 g/l) during 14 days; 4 group – mice that after drinking the aqueous extract of *Auricularia auricular-judae* (during 14 days) were given

a single injection (i.p.) of urethane (1 mg/kg); 5 group – mice were allowed to drink an aqueous extract of *Flammulina velutipes* (4 g/l) for 14 days; 6 group – mice that after drinking the aqueous extract of *Flammulina velutipes* (during 14 days) were given a single injection (i.p.) of urethane (1 mg/kg). In 20 weeks after injecting urethane, mice were sacrificed via cervical dislocating. Extracted lungs were preserved in 10-% formalin. After counting adenomas, the ratio of "adenomas per mouse" and carcinogenesis rate (%) were estimated.

The antitumoral properties of *Flammulina velutipes* were estimated as the rate of induced adenomas in mouse lungs after drinking its aqueous extract (12 g/l) and external irradiation (total dose 7 Gy – twice in 3.5 Gy (^{137}Cs) with a monthly interval): the aqueous extract was used during the 1st month before the first irradiation and after that up to the end of the experiment. The radioprotective properties while drinking the aqueous extract of *Phallus impudicus* were investigated in the following trials: *Af* mice of the control and experimental groups were exposed to a single total γ-irradiation dose of 7.0 Gy (^{137}Cs). After irradiation, all groups of mice were kept on a standard ration in the vivarium. Male mice in the experimental groups drank only the aqueous extract *Phallus impudicus* (4 g/l): (1) during 7 days before irradiation; (2) during 7 days after irradiation. The intensity of the clinical presentation of acute radiation sickness was studied on the following parameters: the survival rate of the irradiated mice and the dynamics of body mass change (body weight measurement once in the first 3 days, the first one was in 3 days after irradiation). An estimation of endogenous colony-formation was carried out on sexually mature mice of both sexes 9 days after total ionizing radiation in doses 5 and 5.5 Gy. During 14 days before and after irradiation, a half of all experimental mice consumed the aqueous extract of dried *Phallus impudicus* (1 g/l) despite drinking water. Extracted spleens were preserved in Telesnitsky liquid (5 ml of formalin + 5 ml of glacial acetic acid + 90 ml of 70 % ethanol) and the number of endogenous spleen colonies was counted.

5.2.3 Results

While studying the influence of the aqueous mushroom extracts on the level of spontaneous and urethane-induced carcinogenesis in *Af* mice lungs, it was established that in animals who drank the aqueous extract of *Auricularia auricular-judae* for 14 days, the number of spontaneous adenomas in lungs over 20 weeks was almost equal to the levels in intact control mice (Table 5.4). The injection of urethane to intact animals resulted in a significant increase of the number of adenomas. Therefore, the ratio of "adenomas per mouse" averaged 17.0 ± 2.7, and tumors were noted in all animals. Administration of the *Auricularia auricular-judae* extract before the urethane lowered the number of adenomas by threefold in one animal in comparison with group "urethane" ($p < 0.05$). After the urethane intoxications, adenomas were registered in 100 % of the animals. Having drunk the aqueous extract of *Flammulina velutipes* within 14 days, the mice had the quantity of adenomas in lungs lowered in

Table 5.4 Number of adenomas in mice lungs as a function of drinking the aqueous extract of *Auricularia auricular-judae* and *Flammulina velutipes* during 20 weeks after injection of urethane

Group	Number of animals	Adenomas per mouse	Carcinogenesis rate (%)
Control	12	0.33 ± 0.21	33.3
Urethane	12	17.0 ± 2.7*	100
Auricularia auricular-judae	12	0.33 ± 0.21	33.3
Auricularia auricular-judae + urethane	16	5.5 ± 0.9**	100
Flammulina velutipes	17	0.12 ± 0.08*	11.8
Flammulina velutipes + urethane	10	11.1 ± 3.1	90

Note: *$p < 0.05$ vs. control; **$p < 0.05$ vs. "urethane" group

Table 5.5 Number of lung adenomas in mice after drinking the aqueous extract of mushroom *Flammulina velutipes* before and after external irradiation in total dose 7 Gy

Group	Number of animals (♂и♀)	Adenomas per mouse	Carcinogenesis rate (%)
Control	30	0.13 ± 0.04	13.3
Irradiation	26	0.36 ± 0.05*	38.5
Flammulina velutipes	28	0.07 ± 0.04	7.1
Flammulina velutipes + irradiation	22	0.18 ± 0.06**	18.2

Note: *$p < 0.05$ vs. control; **$p < 0.05$ vs. "irradiation" group

20 weeks in a statistically significant manner in comparison with control levels ($p < 0.05$) (Table 5.4) as well as the carcinogenesis rate (percent of mice with adenomas). Having drunk the aqueous extract of mushroom *Flammulina velutipes* within 14 days, the animals after injecting the oncogene promotor urethane had the essentially increased average "adenomas per mouse" in comparison with levels in the intact control. However, it was lower than in animals that drank the potable water and were injected with urethane (Table 5.4). Notably, after the preventive effects of the aqueous extract of this mushroom, the frequency of cases with neoplasm decreased by 10 %. Thus, the aqueous extracts of the investigated mushrooms exert an inhibiting influence upon spontaneous and induced oncogenesis in mice lungs.

The external γ-irradiation induced an increase in the quantity of adenomas per mouse as well as the percent of animals with fixed adenomas in *Af* mice lungs, in comparison with levels in the intact control (Table 5.5).

After drinking the aqueous extract of mushroom (beginning 1 month before the first irradiation and before the end of the experiment), the irradiated mice had almost half the number of adenomas per mouse as well as the percent of mice with lung adenomas in relation to parameters of the irradiated mice that drank the water (Table 5.5). It is known that the clinical picture of acute radiation sickness is shown by dyspepsia, bone-marrow changes and other syndromes. Dyspepsia after a single irradiation of mice at a dose 7 Gy was shown by the decrease in the weight of the experimental animals throughout 14–18 days after exposure. In mice that drank the aqueous extract of *Phallus impudicus*, there was a smaller decrease of body weight as well as in its earlier restoration to a normal level (Table 5.6).

Table 5.6 Change of average body weight in mice after a single total irradiation dose of 7 Gy as a function of drinking the aqueous extract

Days after irradiation	Irradiation 7 Gy	Preventive reception of *Phallus impudicus* + irradiation 7 Gy	Irradiation 7 Gy + medical reception of *Phallus impudicus*
4th	16.03 ± 1.91	16.51 ± 1.83	16.28 ± 1.62
7th	15.36 ± 2.11	18.12 ± 1.53*	16.32 ± 2.68
10th	14.37 ± 3.08	17.96 ± 2.08*	15.90 ± 2.15
13th	17.10 ± 4.30	20.35 ± 2.16	15.34 ± 1.93
16th	21.55 ± 3.07	21.49 ± 1.85	19.16 ± 1.74

Note: *$p < 0.05$ vs. control

The use of the aqueous extract of *Phallus impudicus* increased the number of the survived animals. In mice that drank the aqueous extract of the mushrooms within a week after irradiation, the number of surviving animals was 2.7 times higher than irradiated mice that drank water. The greatest positive effect of the use of extract *Phallus impudicus* on animals exposed to irradiation was exhibited in a preventive manner. The survival rate of mice increased by four times when the mice ingested the extract before the irradiation in comparison with the irradiated mice that drank only water. Thus, introduction of the aqueous extract of fruit bodies *Phallus impudicus* to animals decreased the radiation sickness caused by total γ-radiation as shown by the increase of survival rate of mice and the normalization of body weight. Studying regeneration processes of hematopoietic tissue during the postradiating period, Straud and Bruce in 1955 have paid attention to macroscopical small knots, which appeared on a spleen surface through 12–15 days and disappeared on 17 days after the total irradiation of animals with X-rays in a sublethal dose (4–5 Gy). It was the first observation of the phenomena of endogenous colony-formation. Subsequently it has been shown that each colony descends from one parent cell. A colony-forming unit in a spleen (CFUs) thus became a synonym for blood stem cells (BSC). The quantity of spleen colonies formed reflects the state of a BSC pool (the only sources of self-maintenance of all cellular elements of hematopoietic and immune systems) at the moment of an irradiation. Thus, it is possible to test the ability of an extract to stimulate BSC proliferation and to induce radioprotective and hemostimulating effects. As a result of our experiments, it is established that aqueous extracts of *Phallus impudicus* can induce a significant increase in endogenous colonies in the spleen. It may be a consequence of the stimulation of proliferative activity of CFUs [13] (Table 5.7).

On the ninth day after irradiation with a 5 Gy dose, mice that drank the normal drinking water had an average of 2.05 and 1.81 colonies per spleen in males and females, respectively. After ingestion of the aqueous extract of *Phallus impudicus*, the average of colonies per spleen increased by 4.15-fold in males ($p < 0.005$) and by 71.3 % ($p < 0.05$) in females. According to [14], a spleen weight usually correlates well with the number of endogenous colonies and can serve as an additional quantity indicator of the reaction of hematopoietic systems on the stimulating influence. The spleen weight considerably decreased both in males and in females after a total single irradiation. The preventive effects of the aqueous extract of *Phallus*

Table 5.7 Number of endogenous spleen colonies in *Af* mice for 9 days after irradiation in dose 5 Gy

CFUs per spleen		
Irradiation 5 Gy	Irradiation 5 Gy + extract of *Phallus impudicus*	Validity
♂ 2.05 ± 0.44 (n = 19)	4.15 ± 0.48 (n = 20)	$p < 0.005$
♀ 1.81 ± 0.32 (n = 16)	3.09 ± 0.45 (n = 21)	$p < 0.05$

Table 5.8 Spleen weight of *Af* mice for 9 days after irradiation in dose 5 Gy

	Spleen weight, mg		
Groups	Control	Irradiation 5 Gy	Irradiation 5 Gy + extract of *Phallus impudicus*
♂	90.7 ± 3.3	38.0 ± 2.0 (n = 19)	38.0 ± 2.0 (n = 20)
♀	67.8 ± 2.7	26.0 ± 0.7 (n = 16)	36.0 ± 2.0 (n = 21)

impudicus restored spleen weight. On the 9th day after irradiation, the average spleen weight in females exceeded the value in animals that drank water by 38 % ($p < 0.0005$), but in males this effect was not observed (Table 5.8).

Thus, the mushrooms investigated possess antitumoral and radioprotective properties. They can serve as a source to produce medical and prophylactic preparations employed both for stabilizing the state of oncology patients during radiation therapy and for preventing carcinogenesis before the exposure to mutagens.

5.3 Mitigation and Radioprotective Effects of an Exogenous DNA Substance

5.3.1 Introduction

We predicted that small fragments of DNA can protect stem and early progenitor cells from irradiation injury. Our approach is based on scientific knowledge that DNA integration may be an ongoing natural process like the integration of DNA from apoptotic bodies [15] which tightly bind and are accompanied by repair proteins [16]. It is present particularly in cells treated by ionizing irradiation (IR) where double-strand breaks have been appeared. Mechanisms of DNA repair like homologous recombination and non-homologous end joining are implicated in DNA integration [17, 18]. We propose that the exogenous DNA fragments could be transferred in nuclei to be additional templates for DNA repair after irradiation. Improvements in cell survival have been found when irradiated mice receive the exogenous DNA (pattern of 200–6,000 base pairs (bp)) injection [19, 20]. Our research is aimed to find the best method for the preparation of an exogenous DNA substance (exo-DNA

substance), which can protect bone marrow and cardiac tissue using a delay in apoptosis induced by high dose ionizing radiation as a marker of protection.

5.3.2 Materials and Methods

5.3.2.1 Preparation of the Exogenous DNA Substance

For preparation of the "exo-DNA substance", we used fresh porcine liver. The method does not include any toxic chemical agents. The method will be patented.

5.3.2.2 Experimental Model

Male rats (Wistar line, 6-month of age) were used as an animal model. Ionizing irradiation was performed using a γ-(^{137}Cs) source with a working dose of 4.6 microGy/s. Total body irradiation (TBI) was done at a 6.5 Gy sub-lethal dose. Irradiated animals were subcutaneously injected with the test substance during the first hour after irradiation. There were three experimental groups: non-irradiated, irradiated and irradiated and treated animals (n = 3 animals per group). Animals were taken for examination of bone marrow and heart tissue on 3, 10 and 30 days after exposure.

5.3.2.3 Method for the "Micronucleus Test"

The analysis of the frequency of polychromatophilic erythrocytes (PChEr) with micronuclei after the nuclei exclusion stage reflects the unrepaired DNA and abnormal cell differentiation during erythropoiesis. Bone marrow was flushed from tibia bone by phosphate buffered saline (PBS) with 2 % fetal bovine serum (pH 7.4). Cells were put on analytical glasses, fixed by methanol, dried and stained by Giemsa in PBS, than washed by distilled water. The percent of PChEr with micronuclei was calculated for 1,000 polychromatophilic erythrocytes.

5.3.2.4 PCR Analysis of Gene Expression

RNA from the apical part of the heart was isolated using "Aqua Pure RNA isolation kit, Bio-Rad". For the preparation of cDNA, we used "ImProm-II Reverse Transcription System, Promega". Expression of the Bax gene was determined using primer set: reverse primer (3′-antisense):5′-cat ctt ctt cca gat ggt ga-3′; and forward (5′-sense):5′- gtt tca tcc agg atc gag cag-3′, product amplification 487 bp. The control internal gene was glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) gene. It is a glycolytic enzyme with a high and constant gene expression in all

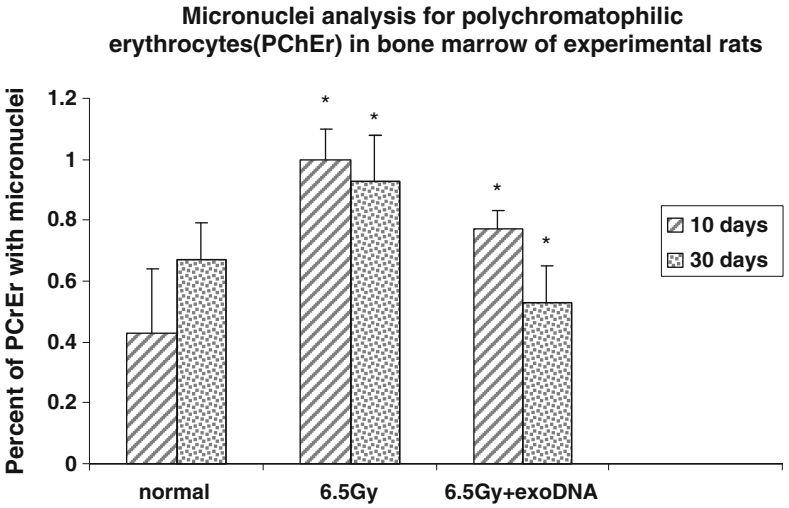

Fig. 5.2 Micronuclei analysis of bone marrow polychromatophilic erythrocytes (PChEr). PCrEr with micronuclei were calculated for 1,000 polychromatophilic erythrocytes and represented as a percent

tissues of mammals. The primer set for GAPDH was: reverse primer (3′-antisense): 5′-ygc ctg ctt cac cac ctt-3′, forward (5′ sense):5′-tgc mtc ctg cac cac caa ct-3′, where m = a or c, y = t or c, product of amplification 349 bp. Primers were produced by "PrimeTech" company, Belarus. PCR kit (M-428) was from "Syntol", Russia. For PCR we used thermocycler "Rortor-Gene, RG 3000", company "Corbett Research", Australia. PCR products were analyzed using the 1.5 % – agarose gel electrophoresis in *tris*-acetate buffer with pH 8 at 75 V during 35 min.

5.3.3 Results and Discussion

5.3.3.1 Effect of Developed Exogenous DNA Substance on Bone Marrow Recovery After Irradiation

The examination of bone marrow was done using micronuclei analysis of polychromatophylic erythrocytes (PChEr). Micronuclei analysis for erythroid progenitors showed an increase in the amount of PChEr with micronuclei by ~2.2 fold in bone marrow of irradiated animals at 10 and 30 days after TBI compared with non-irradiated animals. When irradiated animals received the "exo-DNA substance" injection, we found a decrease in the percentage of micronucleated PChEr in bone marrow compared with the irradiated group at 30 days and this value was further decreased to normal at 30 days (Fig. 5.2). This observation suggests the positive effect of the exo-DNA substance on survival of erythroid progenitors.

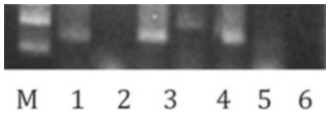

Fig. 5.3 Expression bax gene in cardiac tissues of experimental animals. Animals were divided in three groups: non-irradiated (lines – 1, 2), irradiated at sub-lethal dose (6.5 Gy) (3, 4), and animals received after irradiation the subcutaneous injection of "exo-DNA substance" (5, 6), (n = 3 per case, figure represents one set). Animals were taken to analysis on third day post IR. PCR amplification product for bax is 487 bp, in lines – 2, 4, 6. GAPDH (glycerol-3-phosphate dehydrogenase) gene was used as control gene (amplification product 349 bp), lines – 1, 3, 5 are corresponding to three experimental groups. *M* – DNA molecular markers (500, 250 bp) (1 Kb DNA Ladder, Promega)

5.3.3.2 Effect of the Developed Exogenous DNA Substance on Cardiac Tissue of an Irradiated Organism

The cardiac tissue has been long considered resistant to radiation injury. Our investigation shows that radiation induces apoptosis as identified through the activation of the pro-apoptotic gene Bax in cardiomyocytes. This can ultimately lead to heart failure at late times after irradiation. We examined the expression of the Bax gene as a biomarker of apoptosis in hearts of rats from three experimental groups: non-irradiated animals, total body irradiated at 6.5 Gy and irradiated at 6.5 Gy followed by the single subcutaneous injection of the test "exo-DNA substance" during the first hour after TBI (Fig. 5.3). The level Bax gene expression in normal cardiac tissue was very low (line 2). However, we found the Bax gene was over-expressed in hearts of irradiated animals already after 3 days post IR (line 4). When irradiated animals were treated with the "exo-DNA substance", the expression of Bax was only slightly expressed (line 6). These results suggest that the exo-DNA treatment can protect cardiac cells from Bax gene activation and may represent a promising event for protection of the heart from apoptosis after total body irradiation.

5.4 Conclusion

Thus the studied substances: mixture selenmethionine with methione, water extracts of *Basidiomycetes* and exogenous DNA can be promising agents for the protection and recovery of organisms from radiation injury.

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Part II
Preventing the Harmful Health Effects of Biological Agents

Chapter 6
Homocysteine, Neurotoxicity and Hyperexcitability

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Abstract The research efforts worldwide have established the sulphur-containing amino acid homocysteine (Hcy) as a potent and independent risk factor (or risk marker) for a number of cardiovascular, as well as central nervous system disorders. This vasotoxic and neurotoxic agent interferes with fundamental biological processes and it is metabolized to homocysteine thiolactone, its highly reactive thioester. Hcy and its metabolites induced neuronal damage and cell loss through excitotoxicity and apoptosis. Our results showed that Hcy and Hcy thiolactone significantly affect neuronal cycles, EEG tracings and behavioral responses. After systemic administration, this naturally occurred substance led to the appearance of two different kinds of epileptic activity in adult rats. It has been suggested that Hcy thiolactone may be considered as an excitatory metabolite, capable of becoming a convulsant if accumulated to a greater extent in the brain. It was also found that changes in Na^+/K^+-ATPase activity could be an important factor for the establishment of epileptic focus in Hcy-treated rats. Recently, we demonstrated functional involvement of NO signaling pathway in mechanisms of hyperexcitability caused by Hcy thiolactone. Acute ethanol treatment was shown in our study to decrease EEG power spectra and to represent one of the factors of the exogenous stabilization of brain excitability. Furthermore, our preliminary results showed that hypermethionine diet could contribute to these effects. Developed model of Hcy thiolactone-induced seizures in adult rats allows further investigations of mechanisms involved in Hcy's neurotoxicity and hyperexcitability.

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6.1 Introduction

Within the past several decades, the efforts of researchers have identified the amino acid homocysteine (Hcy) as a potent and independent, "new and emerging" risk factor for arteriosclerosis, as well as vasotoxic and neurotoxic agent involved in fundamental biological processes common to all cells. Therefore, it is also known as "cholesterol of the 21st century".

Total plasma Hcy (tHcy) is defined as the pool of free Hcy, homocystine, Hcy-S-S-Cys disulfide, as well as protein-bound N- and S-linked Hcy, oxidized forms, and Hcy-thiolactone [1–3]. Under physiological conditions, less than 1 % of total Hcy is present in a free reduced form. About 10–20 % of total Hcy is present in different oxidized forms such as Hcy-Cys and homocystine, the Hcy dimer. Plasma tHcy levels are influenced by age, sex and genetic and lifestyle factors, as well as various pathologic conditions [1, 2, 4]. Hyperhomocysteinemia is present when tHcy concentration exceeds 10 μM.

Elevated tHcy is a recognized risk factor for cardiovascular disease [5–8] and has been linked to diseases of the aging brain including cognitive decline, vascular dementia and Alzheimer's disease, cerebrovascular disease and stroke, including epilepsy In addition, Hcy is pro-thrombotic and pro-inflammatory mediator [9, 10].

6.2 Homocysteine Metabolism and Its Implications

Metabolism of Hcy is regulated in order to achieve a balance between the remethylation and transsulfuration pathways which will maintain low levels of this potentially cytotoxic amino acid [11]. Hcy belongs to a group of molecules known as cellular thiols. Glutathione and cysteine, the most abundant cellular thiols, are considered to be "good" thiols, contrary to Hcy [12]. In the methylation pathway, Hcy acquires a methyl group to form methionine in a vitamin B12 dependent reaction catalyzed by the enzyme methionine synthase. The kidney, liver and eye lens have the capacity to convert Hcy to methionine trough a vitamin B12-independent reaction catalyzed by betaine-Hcy S-methyltransferaze (BHMT). On the contrary, the CNS lacks BHMT and therefore conversion of Hcy to methionine is completely dependent on the vitamin B12 and folate pathway.

Hcy condenses with serine to form cystathionine in an irreversible reaction catalyzed by the B6 containing enzyme – cystathionine beta-synthase, known as the transsulfuration pathway. Hcy catabolism requires vitamin B6 and as a consequence, alteration in folic acid and B vitamins status impairs Hcy biotransformation. These alterations result in the in the synthesis of cysteine, taurine and inorganic sulfates that are excreted in urine.

Elevation of Hcy levels is known to lead to metabolic conversion and inadvertent elevation of homocysteine thiolactone, a reactive thioester representing less than 1 % of total plasma Hcy. In all cell types, including endothelial and nerve cells, Hcy is metabolized to homocysteine thiolactone by methionyl-tRNA synthetase [13]. Homocysteine thiolactone causes lethality, growth retardation, blisters and somite

development abnormalities by oxidative stress, one important mechanism for toxicity to neural cells [14]. The highly reactive Hcy metabolite homocysteine thiolactone can be produced in two steps by enzymatic and/or non-enzymatic reactions in blood serum. Therefore, the ability to detoxify or eliminate homocysteine thiolactone is essential for biological integrity [13, 14].

6.3 Contribution of Homocysteine to Neurotoxicity

A number of studies provided evidences for a complex and multifaceted relation of homocysteinemia and CNS disorders Hcy, as endogenous compound, is neurotoxic in supraphysiological concentrations. The hypothesis that relates Hcy to CNS dysfunction is based on its neuroactive properties. Adverse effects on brain functioning and debilities of high tissue Hcy concentrations appeared through oxidative stress and excitotoxicity-induced effects on neurons [15], and together with homocystinuria characterize patients with convulsions [16].

Hcy induces neuronal damage and cell loss through excitotoxicity and apoptosis, what could be a consequence of the inability of cerebral tissue to metabolize Hcy through the betaine and transsulfuration pathways, favoring Hcy accumulation in the nervous system [17]. High brain concentrations of either Hcy or its oxidised derivatives might alter neurotransmission [18]. An accumulation of Hcy (at synapses or in the extracellular space) would increase intracellular S adenosylhomocysteine (SAH), which is a potent inhibitor of many methylation reactions that are vital for neurological function including the O-methylation of biogenic amines. Methylation of myelin basic protein and reducing the synthesis of phosphatidyl choline, which can lead to disruption of the blood-brain barrier (BBB) are possible in absence of normal methylation patterns [19].

According to recent theory, Hcy toxicity is a consequence of covalent binding to proteins, interfering with protein biosynthesis, decreasing normal physiological activity of proteins thus modifying their functions in process called homocysteinylation [13, 20]. Therefore, increased intracellular Hcy concentration is associated with both alteration of redox balance and post-translational protein modifications through *N*- and *S*-homocysteinylation [21]. Moreover, some studies suggest that Hcy induces the expression of superoxide dismutase in endothelial cells, consumption of NO^- and impaired endothelial vasorelaxation [22].

6.4 Hyperexcitability Induced by Homocysteine

6.4.1 Experimental Models of Seizures

Experimental models of epilepsy may be induced by manipulation of γ-aminobuturic acid (bicuculline, corasol, picrotoxin, benzylpenicillin sodium) [23] or by increasing cerebral excitatory neurotransmission. Experimental rat models of generalized

clonic-tonic seizure induced by metaphit [24, 25] and lindane [26–28] are suitable for the studies of epilepsy and preclinical evaluation of potential antiepileptic treatments. Almost four decades ago, Sprince et al. [29] described that high levels of Hcy, arising from excess dietary methionine, may induce epilepsy and lethality.

The fact that the elevated Hcy concentrations persists in damaged endothelial structures, during aging and antiepileptic-drug-therapy [30] justifies attention directed towards the examinations of homocysteine thiolactone effects. Namely, classical anticonvulsants (phenytoin, carbamazepine and valproic acid) lower plasma folate levels and increased significantly Hcy levels inducing epileptogenic brain and suboptimal control of seizures in the patients with epilepsy [31].

6.4.2 Two Types of Seizures in Adult Rats upon Homocysteine Thiolactone Administration

Stanojlovi et al. [32] suggested that D, L-homocysteine thiolactone may be considered as an excitatory metabolite, capable of becoming a natural convulsant if accumulated to a great extent in the brain. Hyperhomocysteinemia in awake adult Wistar male rats induced recurrent unprovoked clonic-tonic convulsions and absence-like seizures, as well as specific electrical discharges. The seizure incidence, median seizure episode severity, median number of seizure episodes per rat, was significantly higher in all Hcy treated groups together with prolonged median latency to the first seizure [32]. Non-convulsive status epilepticus can occur from variety of causes including primarily generalized absence epilepsy, genetic origins (Wakayama or tremor epileptic rats) or pharmacologically (penicillin, pentylenetetrazole, γ-hydroxy-butyric acid) induced models [33]. The most puzzling phenomena in absence epilepsy are behavioral immobility during the active motor cortex and the occurrence of generalized spike-wave activity. SWDs may belong to the same class of phenomena such as sleep spindles. Sleep spindles are normally generated sleep rhythms that transform one, two or more spindle waves into the spike component of the SWD [34].

According to well known fact that rhythmic bursts of spikes represent an electrophysiological marker of a hyperexcitability, Folbergrova et al. [35] found very poor electroclinical correlation together with dissociation between electroencephalographic (EEG) pattern and motor phenomena in immature rats. The epileptogenic process is closely associated with the changes in neuronal synchronization. Non-lesion, non-convulsive, generalized epilepsy is characterized by brief episodes of unpredictable and unresponsive behavior with a sudden arrest accompanied by SWDs. This second type of spike-wave complexes had different shape. Bilateral, high-voltage synchronous, spindle-like electrical oscillations, phenomenon of paroxysmal electroencephalographic attacks, termed SWDs were associated with a sudden motor immobility and minor clinical signs like loss of responsiveness with rhythmic twitches of vibrissae or cervicofacial musculature were seen after i.p. administration of D, L-homocysteine thiolactone in adult rats.

Stanojlović et al. [32] found poor electro clinical correlations and dissociation of activity in rats. It is worth mentioning that electrographic seizure discharge was absent even during motor convulsions of grade 3 or 4, while on the contrary, EEG seizures without motor symptoms were regularly observed. Foremention EEG graphoelements were distinguishable from sleep spindles (10–16 Hz), regarding their frequency, duration, morphology (sleep spindles are more stereotyped than SWD waves) and moment of occurrence (SWD occurs during passive wakefulness *vs*. sleep spindle-like oscillation occurring during high amplitude delta activity.

6.4.3 Mechanisms of Homocysteine Convulsive Effects

There are several proposed mechanisms by which exposure to excess D,L-homocysteine thiolactone induces seizure [36].

Increased levels of Hcy and its metabolites could provoke seizures by increasing activation of some receptors like N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainite ionotropic glutamate receptors [1]. These receptors are expressed in hippocampal pyramidal cells and may directly induce or drive these cells over the threshold for excitotoxic cell death. Overstimulation of these receptors triggers Ca^{2+} influx and intraneuronal calcium mobilization in the presence of glycine [37]. Increased cytosolic Ca^{2+} concentrations affect enzyme activities and synthesis of nitric oxide [38]. It should be noted that expression of NMDA receptor is not confined to neurons. Other cells, including endothelial cells from cerebral tissue, can express this receptor. Free radicals induce up-regulation of the NR1 subunit of the NMDA receptor, increasing the susceptibility of cerebral endothelial cells to excitatory amino acids, favoring BBB disruption [39]. Also, microglia is subject to toxic effects of Hcy [40]. Hcy could induce convulsions in adult, as well as in immature experimental animals throw modulating the activity of metabotropic glutamate receptors (mGluRs) [41].

Hcy was shown to enhance either the release or uptake of other endogenous excitatory amino acids [41]. It seems that Hcy exerts a direct excitatory effect comparable to the action of glutamate [16].

Rasic-Markovic et al. [42] investigated the effects of MK-801, NMDA anatagonis, as well as, of ifenprodile, NR2B-selective NMDA antagonist in homocysteine thiolactone seizures and showed involvement of this mechanisms in homocysteine thiolactone induced epileptogenesis.

6.4.4 Involvement of nNOS Signaling Pathways in Homocysteine Hyperexcitability

Nitric oxide (NO) is a highly reactive messenger molecule synthesized in a number of tissues with key role in new form of interneuronal communication via modulating release of classical neurotransmitters and excitability status of neurons.

Hrnčić et al. [43] determined the role of NO in mechanisms of D, L homocysteine thiolactone induced seizures by testing the action of L-arginine (NO precursor) and L-NAME (NOS inhibitor) on behavioral and EEG manifestations of D, L homocysteine thiolactone induced seizures. Recently, the involvement of neuronal NO synthase (nNOS) in homocysteine thiolactone – induced seizures was determined using pharmacological inhibition of this enzyme by 7-nitroindazole, its selective inhibitor [44]. Congruent results with those obtained using non-selective inhibition were obtained.

6.4.5 Homocysteine and Na^+/K^+ATPase Activity

The function of the Na^+/K^+-ATPase is essential for generation of the membrane potential and maintenance of neuronal excitability [12]. Rašić-Marković et al. [45] demonstrated a moderate inhibition of rat hippocampal Na^+/K^+-ATPase activity by D,L-homocysteine, which however expressed no effect on the activity of this enzyme in the cortex and brain stem. In contrast, D,L-homocysteine thiolactone strongly inhibited Na^+/K^+-ATPase activity in cortex, hippocampus, and brain stem of rats structures affecting the membrane potential with deleterious effects for neurons. Hrnčić et al. [43] demonstrated that L-Arginine when applied alone, significantly increases the activity of Na^+/K^+-ATPase activity in the hippocampus, the cortex and the brain stem and when applied prior to homocysteine thiolacotne completely reversed the inhibitory effect of homocysteine thiolactone.

6.4.6 Modulation of Homocysteine-Induced Hyperexcitability

Complex relationship between sleep and epilepsy is still of special interest for neuroscientists since neurophysiological basis of that relation is far from being completely understood [46]. Sleep is a cyclic vital physiological process that makes one-third of human life [47]. It is estimated that about 20 % of the world's population still suffer from decrease in sleep time due to change of lifestyle and sleep disorders as the major causes. Recently, we have shown aggravation of seizure activity in homocysteine thiolactone – treated rats upon selective REM sleep deprivation [48].

Rašić-Marković et al. [49] examined the changes of total spectral power density in adult rats after ethanol alone and together with homocysteine thiolactone and it was found that action of ethanol on electrographic pattern was biphasic, with potentiation of epileptiform activity in one dose range and depression in another one. Low ethanol doses causing euphoria and behavioral arousal are associated with desynchronization of the EEG, decrease in the mean amplitude, and increase in the theta and alpha activity. Ethanol increased mean total spectral power density 15 and 30 min after administration, in all ethanol groups.

6.5 Conclusion

Results of aforementioned studies demonstrated that acute administration of homocysteine thiolactone significantly affected neuronal cycles, EEG tracing and behavioral responses. After systemic administration, this natural substance led to the disturbances in brain functioning and to the appearance of two different kinds of neuron network in adult Wistar rat males. It could be supposed that hyperhomocysteinemia might express similar effects on human brain activity. These effects are connected with stimulation of NMDA receptors, inhibition of the Na^+,K^+-ATPase activity, and NO mediated signaling pathways during. Modulation of homocysteine – induced hyperexcitability was achieved by REM sleep deprivation and ethanol administration.

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Chapter 7
Oxidation of Selected Lipids in Low Density Lipoprotein: Effects on Calcium Transients in Isolated Rabbit Cardiomyocytes

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Abstract When LDL is oxidized by a free radical generating system, both cholesterol and fatty acyl moieties within the LDL are modified. Oxidized low density lipoprotein (oxLDL) can induce alterations in the Ca^{2+} transients in isolated cardiomyocytes. It is unclear if oxidation of the LDL fatty acyl chains (in phospholipids, triglycerides and cholesteryl esters) or oxidation of the LDL unesterified cholesterol is more important in producing the oxLDL-induced alteration in cellular calcium transients. Therefore, we investigated the possible role of oxidized cholesterol and fatty acyl chain peroxidation in the effects of LDL on Ca^{2+} transients. Cholesterol oxidase (CO) treatment of LDL produced oxidized cholesterol plus H_2O_2. The H_2O_2 peroxidized the LDL fatty acyl chains, as indicated by an increased malondialdehyde (MDA) content. The cell systolic [Ca^{2+}] was significantly increased after incubation with CO-treated LDL. Diastolic [Ca^{2+}] was unchanged. MDA content in the CO-treated LDL correlated with the change in systolic [Ca^{2+}] of treated cells. Catalase, a scavenger of H_2O_2, inhibited MDA formation in the CO-treated LDL and prevented the increment in systolic [Ca^{2+}] in the treated cells. A similar stimulatory effect on the Ca^{2+} transient was observed if cells were treated with LDL after exposure to only H_2O_2 and not CO.

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Direct exposure of myocytes to H_2O_2 (without LDL) failed to produce a stimulatory effect on the calcium transient but caused an increment in cellular diastolic Ca^{2+}. Exposure of myocytes to CO alone (without LDL) produced a significant increment in diastolic [Ca^{2+}] and this effect was not prevented by catalase. These results suggest that fatty acid peroxidation in the LDL moiety is more important than oxidized cholesterol in the generation of ox-LDL-induced increases in Ca^{2+} transients in isolated cardiomyocytes. Further, oxidation of in situ cell membrane cholesterol will destroy cell integrity. Our data also underline the importance of adding extracellular lipid in any study of the effects of oxygen free radicals on cellular function. Under conditions of radiologic or chemically-induced generation of oxygen-derived free radicals, cardiac dysfunction and damage may be induced by this process.

7.1 Introduction

The generation of oxygen-derived free radicals is proposed to increase during hypoxia/reoxygenation and ischemia/reperfusion injuries to the heart [3, 4, 22]. Chemical, biological and radiological conditions can also induce damaging and even lethal levels of oxygen-derived free radicals. One of the targets of the free radicals is thought to be the cardiomyocyte itself. Free radicals increase the resting intracellular [Ca^{2+}] and thereby contribute to cell damage and death [6, 20]. However, another important target of the free radicals is circulating lipids like LDL (low density lipoprotein). LDL can be extensively oxidized by free radicals [38]. Since LDL is in contact with cardiomyocytes in relative high concentrations [21], and free radicals present in the interstitial space may have been shown to oxidize LDL there [15, 40, 46], it is possible that oxidized LDL (oxLDL) may exert an effect of its own on the cardiomyocytes. Recently, we observed that oxLDL can significantly alter the Ca^{2+} transient in isolated cardiomyocytes [27]. The oxLDL greatly potentiated the effects of free radicals on their own. In view of the significance of free radical-induced damage to the heart [3, 4, 22], and the potential role of ox-LDL as a mediator in this action, it was important to investigate the mechanism whereby oxLDL exerts its effects on the cardiomyocyte Ca^{2+} transients.

The purpose of the present study was to investigate which part of the oxLDL particle is most important for producing the effects of oxLDL on the Ca^{2+} transient of the isolated cardiomyocyte. Three major parts of the LDL lipoprotein moiety (apoprotein, fatty acyl chain and cholesterol) can be oxidized [1, 42]. If the fatty acyl chain present in LDL phospholipids, cholesterol esters and triglycerides is oxidized extensively, a broad spectrum of shorter chain aldehydes (conjugated dienes, malondialdehyde and 4-hydroxynonenal) is generated [11, 17, 42]. The unesterified cholesterol in LDL can also be oxidized into several cholesterol oxide derivatives [1, 28, 29]. Currently, it is difficult to evaluate which lipid in the oxLDL moiety plays the most important role in modifying the Ca^{2+} transport characteristics [24]. We decided, therefore, to examine if oxidized cholesterol in LDL may

be responsible for the effects of oxLDL on the cardiomyocyte Ca^{2+} levels. In the present investigation, cholesterol oxidase was employed to specifically oxidize the cholesterol in the LDL.

7.2 Materials and Methods

7.2.1 Materials

All chemicals were of standard reagent grade (Sigma Chemical Co., St. Louis, MO). Cholesterol oxidase (CO) (*Pseudomonas fluorescens*) and catalase (bovine liver) were obtained from Sigma Chemical Co. Other selected cholesterol derivatives which were used as standards for the high performance liquid chromatography (HPLC) analysis were purchased from Steraloids, Inc., Wilton, NH. Fura-2AM was purchased from Molecular Probes Inc., Junction City, Oregon. Collagenase was obtained from Worthington Biochemicals (Freehold, New Jersey). The cholesterol supplemented rabbit chow was purchased from ICN Biochemicals Inc. (Cleveland, OH). Lazaroid (U74500A) was kindly provided by The Upjohn Co.

7.2.2 Cardiomyocyte Isolation

Calcium tolerant, contractile, single cardiomyocytes were prepared according to a procedure which has been described in detail previously [26, 30]. In brief, the hearts from 2.5 kg male albino New Zealand rabbits were quickly excised, the ascending aorta cannulated, and the heart retrogradely perfused with a Ca^{2+}-free then minimal Ca^{2+} containing solution supplemented with 1.3 mg/ml collagenase and 0.5 mg/ml hyaluronidase. The heart was then removed from the isolation apparatus after ~30 min perfusion and the cells gently minced free in a petri dish. Myocytes were harvested at 1 xg and viable cardiomyocytes adhered to coverslips which had been previously coated for ~20 min with a 0.2 mg/ml laminin. More than 70 % of the myocytes were rod-like shaped, calcium-tolerant, free of membrane blebs, possessed clear sarcomere striation, did not spontaneously contract and excluded trypan blue. Myocytes were studies in a Kreb's perfusion solution containing (in mM): NaCl 120; $NaHCO_3$ 25, KCl 4; KH_2PO_4 1.2; $MgSO_4$ 1.2; dextrose 5.5; $CaCl_2$ 1.8; (pH 7.4). All experiments were performed at a temperature of 37 °C.

7.2.3 Lipoprotein Isolation

LDL (density 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation from the plasma of male albino New Zealand rabbits fed a 1 % cholesterol-supplemented diet [34]. Dithiobisnitrobenzoic acid (1.5 mM), phenylmethylsulphonyl fluoride

(2 mM), thimerosal (0.08 mg/ml) were added to the plasma after separation of the blood cells to inhibit lecithin:cholesterol acyl transferase, proteolysis and bactericides, respectively [25]. Ascorbic acid (50 μM) and EDTA (1 mM) were added throughout the isolation to prevent oxidation of LDL [25, 30]. The LDL fraction was extensively dialyzed against 0.15 M NaCl, 1 mM EDTA (pH 7.4) and stored at 4 °C. The protein content of LDL was determined by Lowry's method [32], and cholesterol (free and esterified) was measured enzymatically as described [37]. The absence of LDL oxidization during isolation or prior to their use in experiments was estimated by an absence of malondialdehyde (MDA) reactive products [13, 27, 28] and oxidized cholesterol [28, 31, 41].

7.2.4 Preparation and Measurement of Lipid Oxidation

Lipoprotein oxidation was induced by incubation with CO ± catalase at 37 °C for 60 min. CO and catalase concentrations used were 1 mg/ml each throughout the experiments or as otherwise indicated. An aliquot of oxidized LDL was then applied to the medium bathing the experimental myocyte (tenfold dilution). Lipid peroxidation of LDL was assessed by measuring the MDA content of LDL using the thiobarbituric acid reactive substances (TBARS) method [13, 28]. Freshly diluted malondialdehyde bis (dimethyl acetal 1,1,3,3-tetra-methoxypropane) was used as a reference standard. TBARS were expressed as MDA equivalents. Oxidized cholesterol species in myocytes treated with oxLDL were identified and quantified using HPLC. A modification of the technique of Sevanian and McLeod [41] was employed, as described in detail elsewhere (18, 24, 28). Briefly, myocytes were treated with various agents in M199 solution for 60 min at 37 °C followed by washing the cells three times. The treated cells were then homogenized in a solution containing 0.5 % Triton X-100, 3 mM sodium cholate and 0.1 M Tris (pH 6.6), transferred to 5 ml of 2:1 chloroform:methanol, and extracted overnight at 4 °C in the dark. After the samples were lipid extracted as described in detail elsewhere [24, 28], the lipids were then suspended in 20 μl of methylene chloride of which 10 μl was used for HPLC analysis. The HPLC used included a Waters 501 HPLC pump, 116 solvent delivery system, Waters 484 tunable absorbance detector and a Waters Nova-Pak Silica column (3.9 × 150 mm). Flow rate was kept at 2.0 ml/min and the mobile phase was 99:1 hexane:isopropanol. UV detection of the peaks was carried out at an absorbance of 208 nm. The Waters Baseline 810 chromatography workstation was employed in operating the process.

7.2.5 Measurement of Cellular Ca^{2+} Transients

The fluorescent Ca^{2+} indicator dye fura-2 was used to measure intracellular Ca^{2+} transients [10, 18]. Briefly, myocytes were loaded with 2 μM fura-2AM (acetoxymethylester) for 15 min at 22 °C, then were gently washed twice with Kreb's solution.

These cells were then studied in a chamber on the stage of a Nikon Diaphot epifluorescent microscope which was attached to a SPEX Fluorolog spectrofluorometer (SPEX Industries, Edison, NJ) [10, 27, 30]. Cardiomyocytes were sequentially excited at 340 and 380 nm wavelength light and the emitted fluorescent images of the myocyte at 505 nm were recorded and quantitated with photomultiplier tubes coupled to a computer. Calibration of the monocytes' fluorescent ratio was carried out with the in situ method of Grynkiewicz et al. [14] using 20 μM 4-bromo-A23187 to obtain maximal fluorescence and 10 mM EGTA to determine the minimum fluorescence [6, 14].

For each experiment, a single cardiomyocyte was selected under the phase contrast bright field microscopy and then stimulated by passing currents via two platinum electrodes spaced ~8 mm apart. The cell contracted in response to this field stimulation which was set at a frequency of 0.5 Hz with biphasic pulses of 3 ms in duration. Stimulation voltage across the electrodes as determined in air was 8–12 V.

7.2.6 Statistical Analysis

All results are expressed as mean ± SEM. Data was analyzed statistically with one-way analysis of variance followed by a Duncan's *post hoc* test [25]. The level of significance was arbitrarily set at $P < 0.05$.

7.3 Results

LDL was pre-incubated with 0.1 mg/ml CO in the absence of catalase. The incubation of LDL with CO alone will result in an oxidation of LDL cholesterol and the generation of H_2O_2. An aliquot was removed and placed in a solution bathing the cardiomyocytes and the intracellular calcium transient of isolated cardiomyocytes was measured. In the control cells, the Ca^{2+} transient is stable for more than 60 min of stimulation [30]. Fig. 7.1 shows representative results of calcium transients over time in cardiomyocytes incubated with CO treated LDL ± catalase. Recordings were paused after 25 s every 2 min in order to avoid photo bleaching the dye which could have occurred during long exposure times to light. This protocol was used throughout the experiments. A slow and steady increment in the Ca^{2+} transient was observed after oxLDL was incubated with the cardiomyocyte (Fig. 7.1a). A striking increment in peak systolic [Ca^{2+}] during the transient was noticeable during the treatment whereas the diastolic Ca^{2+} level was unaltered. An inexcitability of treated cells was consistently observed after ~16 min treatment with oxLDL (Fig. 7.1a). When the above treatment was applied to cells but in the presence of 0.1 mg/ml catalase to in-activate the H_2O_2 (Fig. 7.1b), the amplitude of the Ca^{2+} transient was not significantly increased. A number of experiments were carried out to investigate the effects of varying concentrations of oxLDL ± catalase on the Ca^{2+} transients in cardiomyocytes (Table 7.1). The systolic level of the Ca^{2+} transients was not significantly increased

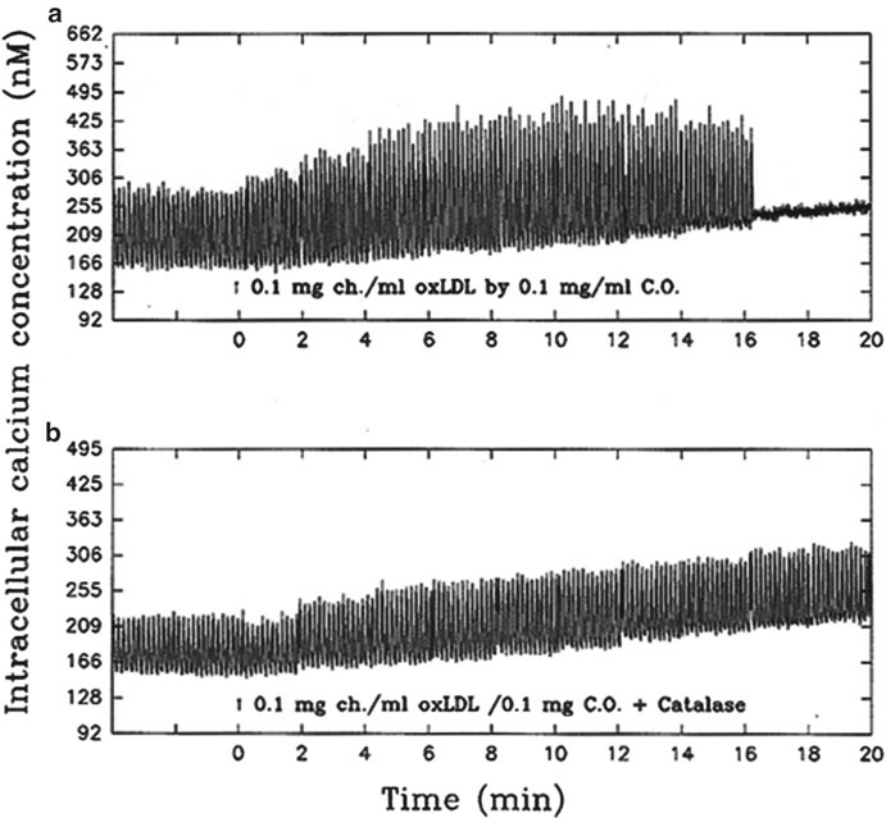

Fig. 7.1 Representative recordings of calcium transients in cardiomyocytes treated with 0.1 mg cholesterol/ml LDL oxidized by 0.1 mg/ml cholesterol oxidase (CO) (**a**) or 0.1 mg cholesterol/ml LDL oxidized by 0.1 mg/ml CO in the presence of 0.1 mg/ml catalase (**b**). The recordings here are not continuous but for 25 s then stopped. At the second minute, another 25 s recording was resumed, followed by another 25 s recording at the fourth minute, etc. Note the increase in the size of calcium transient induced after the cell was exposed to 0.1 mg/ml oxLDL treatment (**a**) and the relative stability in the calcium transient recording in the cell treated with 0.1 mg/ml oxLDL but in the presence of 0.1 mg/ml catalase (**b**)

until the oxLDL concentration reached 50 μg cholesterol/ml oxLDL. This effect required at least 16 min before statistical significance was achieved. This effect was not observed when catalase was present during the treatment of oxLDL. The diastolic [Ca^{2+}] was not significantly altered at any of the concentrations applied.

To understand the mechanism of action of oxLDL on the Ca^{2+} transient of treated cardiomyocytes, the association of lipid peroxidation within the LDL to the change in the intracellular Ca^{2+} transient was investigated. MDA products within the LDL were determined as an index of lipid peroxidation. A good correlation between the oxLDL MDA content and the percentage increase in systolic Ca^{2+} transient was observed in Fig. 7.2.

Table 7.1 The effect of varying concentrations of cholesterol oxidase treated LDL ± catalayse on calcium transients in rabbit ventricular myocytes

	Time (min)				
CO treated [LDL] ± catalase	0	4	8	12	16
	nM [Ca^{2+}]				
10 μg/ml					
Systolic	207 ± 13	209 ± 11	205 ± 14	208 ± 11	208 ± 12
Diastolic	117 ± 9	117 ± 7	121 ± 7	119 ± 11	124 ± 9
50 μg/ml					
Systolic	224 ± 25	243 ± 30	276 ± 20	310 ± 38	355 ± 52 *
Diastolic	114 ± 16	114 ± 16	128 ± 24	132 ± 27	133 ± 28
50 μg/ml + catalase					
Systolic	238 ± 25	242 ± 27	252 ± 26	256 ± 25	260 ± 21
Diastolic	155 ± 25	156 ± 24	162 ± 23	172 ± 27	181 ± 28
100 μg/ml + catalase					
Systolic	235 ± 49	287 ± 62	326 ± 61	355 ± 73	383 ± 39 *
Diastolic	147 ± 42	169 ± 45	183 ± 46	207 ± 52	227 ± 51
100 μg/ml + catalase					
Systolic	169 ± 23	181 ± 20	202 ± 21	208 ± 29	221 ± 27
Diastolic	98 ± 6	103 ± 7	118 ± 14	119 ± 15	127 ± 24

Notes: Values represent mean ± S.E.M. (n = 4–8) *P < 0.05 vs. control. If included, catalase was present at ratio of 1:1 (mg/mg) with cholesterol oxidize. Calcium concentration is in nM

The efficacy of antioxidants in preventing the effects of cholesterol oxidase on LDL was compared. Catalase I in a ratio of 1:1 (w:w) to cholesterol oxidase completely inhibited MDA formation in the oxLDL (Fig. 7.3a). Lazaroid, a novel antioxidant, and vitamin E also prevented the production of MDA in the oxLDL but less efficiently compared to catalase (Fig. 7.3b).

Cholesterol oxidase (CO) itself may have a strong influence on the cardiomyocyte Ca^{2+} transient. Therefore, in order to exclude the possibility that CO itself may be inducing a direct effect rather than the oxLDL, the effect on the Ca^{2+} transient of varying the concentration of CO ± catalase was examined in the absence of LDL (Fig. 7.4, n = 4–6). Figure 7.4 shows representative calcium transients in the cardiomyocytes after treatment with 1 mg/ml cholesterol oxidase (CO) (A), 1.5 mg/ml CO (B), 2 mg/ml CO (C), 0.5 mg/ml catalase (D) and 2 mg/ml CO + 0.5 mg/ml catalase (E). Recordings were only for 25 s, then stopped and the data saved before reinitiating the recording again for 25 s two minutes later. The CO was added at the time point indicated by the arrow. Note the stability in the calcium transient recorded from cells treated with 1 mg/ml CO (A) and the striking elevation in the diastolic component of the calcium transient induced by 1.5 and 2 mg/ml CO treatment (B and C). Note the steadiness of Ca^{2+} transient in the cells treated with 0.5 mg/ml catalase (D) and the complete loss of excitability in the cells treated with 2 mg/ml CO + 0.5 mg/ml catalase (E). When 1 mg/ml CO was applied to cardiomyocytes, no noticeable alteration in the myocyte Ca^{2+} transient was observed except an inexcitability appeared at 18 min (Fig. 7.4a). However, when the CO concentration was

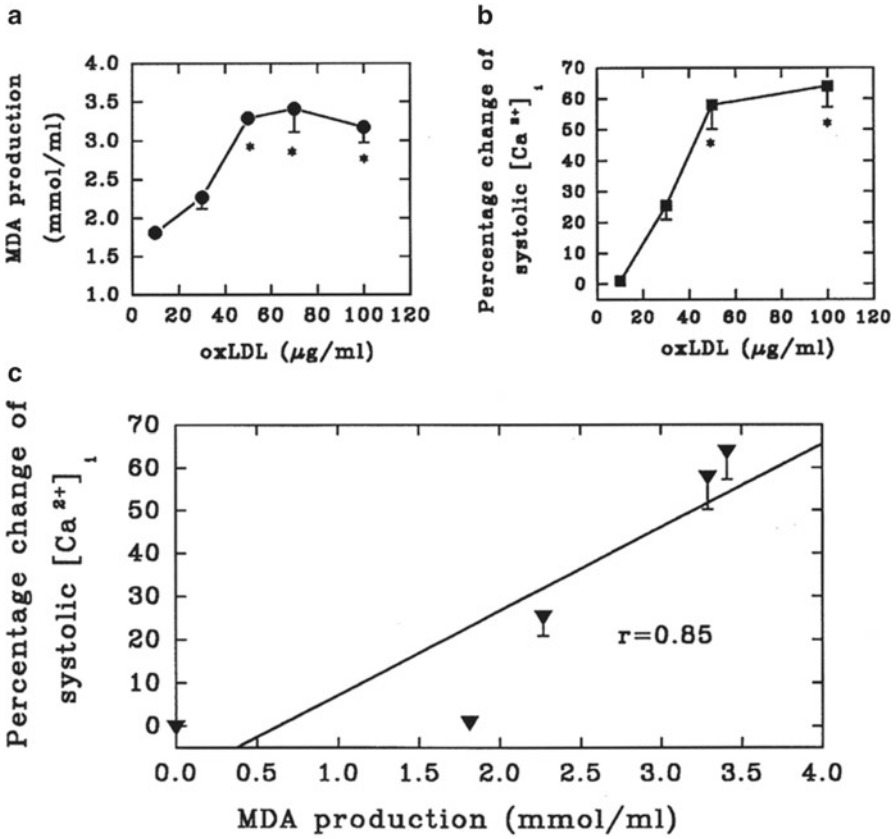

Fig. 7.2 The effects of varying the oxLDL concentration on the cellular calcium transient and the LDL MDA content. *Panel* **a** shows the MDA production as a function of the oxLDL concentration. *P < 0.05 vs. MDA value at 10 μg/ml LDL. *Panel* **b** shows the percentage change in the systolic calcium transient vs. oxLDL concentration. *P < 0.05 vs. control untreated LDL. Values represent the mean ± S.E.M. from 4 to 6 experiments using different cells in both panels. *Panel* **c** presents the correlation between the MDA content of oxLDL and the percentage change of systolic calcium transients in cardiomyocytes treated with oxLDL

increased to 1.5 mg/ml, a striking increment in the diastolic component of the Ca^{2+} transient of treated myocytes was observed (Fig. 7.4b). An immediate increment in the diastolic component of the Ca^{2+} transient appeared when the CO concentration applied to the cardiomyocyte was increased to 2 mg/ml (Fig. 7.4c). The possible protective role of catalase against this effect produced by CO was also examined. The Ca^{2+} transient of cardiomyocytes remained stable after 0.5 mg/ml catalase was incubated with cardiomyocytes for 20 min (Fig. 7.4d). However, catalase at this concentration failed to protect against the effects of 2 mg/ml CO on the cell Ca^{2+} transient (Fig. 7.4e).

HPLC was used to determine if CO was indeed oxidizing cell membrane cholesterol (Fig. 7.5). Cholesterol is a major component of the cell membrane (Fig. 7.5b).

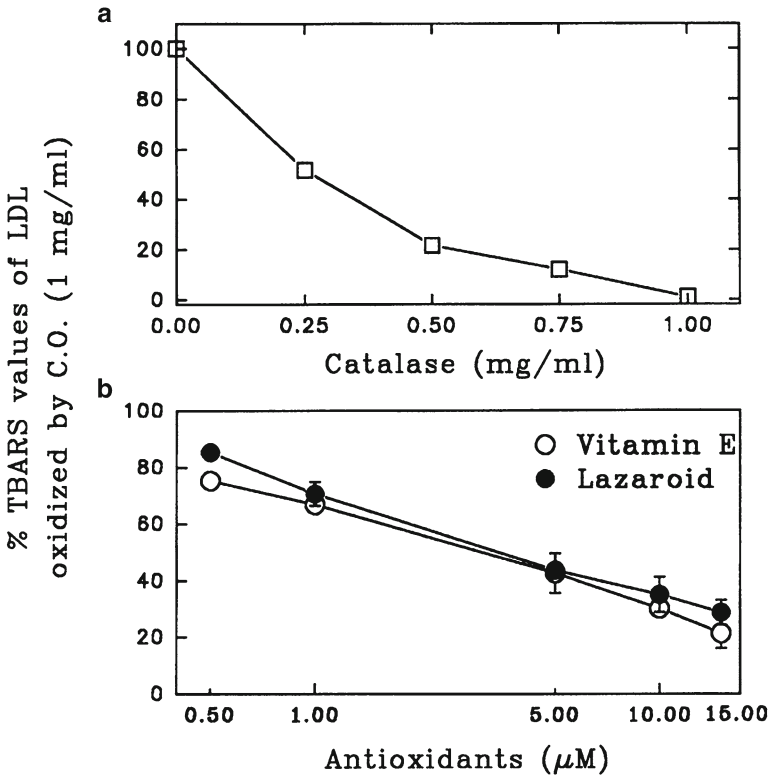

Fig. 7.3 The effects of catalase and other antioxidants on MDA production in oxLDL. *Panel* **a** presents the effect of catalase n the MDA production. *Panel* **b** shows influence of vitamin E and lazaroid on the MDA production. Values are the mean ± S.E.M. from 4 to 6 different experiments. If standard error bars are not present, then the symbol size was greater than the standard error of the mean

One oxidized cholesterol species, 20 α-OH, was also detectable in the control cardiomyocyte. This may be due to the presence of some necrotic cells in the preparation. Our cell isolation yield is about 70–80 % rod shaped, live, viable cells and 20–30 % rounded, necrotic cells (Fig. 7.5b). The most striking change in membrane cholesterol after CO treatment was the appearance of 4-cholesten-3-one (Fig. 7.5c, d). 4-cholesten-3-one became very prominent when the CO concentration was increased to 2 mg/ml and cholesterol disappeared (Fig. 7.5d).

The by-product of cholesterol oxidation by cholesterol oxidase is hydrogen peroxide [43]. The possibility exists, therefore, that the effects on the cardiomyocyte Ca^{2+} transient produced by oxLDL may be due to the presence of H_2O_2 rather than oxLDL itself. Therefore, the effect of varying the H_2O_2 concentration on the Ca^{2+} transient of cardiomyocytes was investigated (Fig. 7.6). A significant increment in the diastolic Ca^{2+} concentration of treated cardiomyocytes was observed. The greater the $[H_2O_2]$ that was applied to the cell, the faster the diastolic $[Ca^{2+}]$ increased

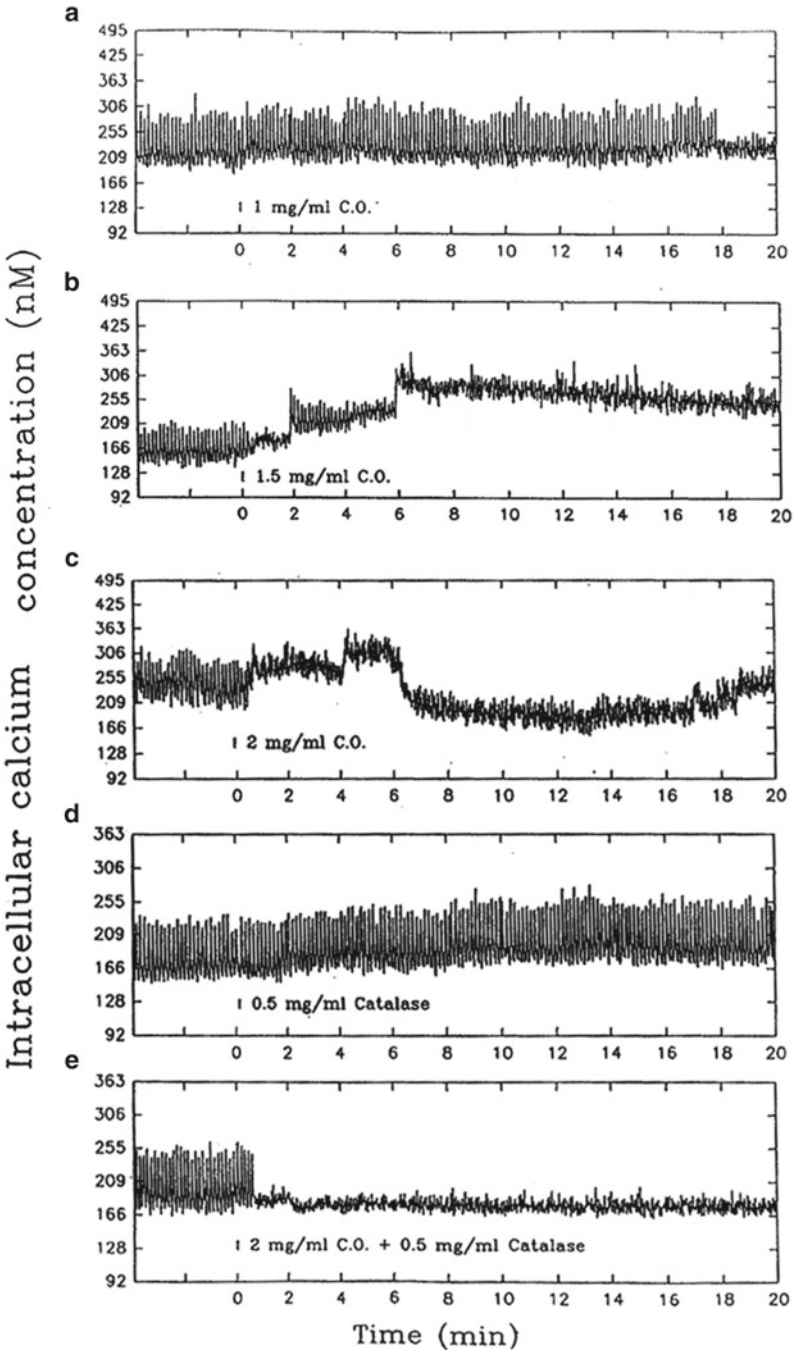

Fig. 7.4 Representative recordings of calcium transients in the cardiomyocytes

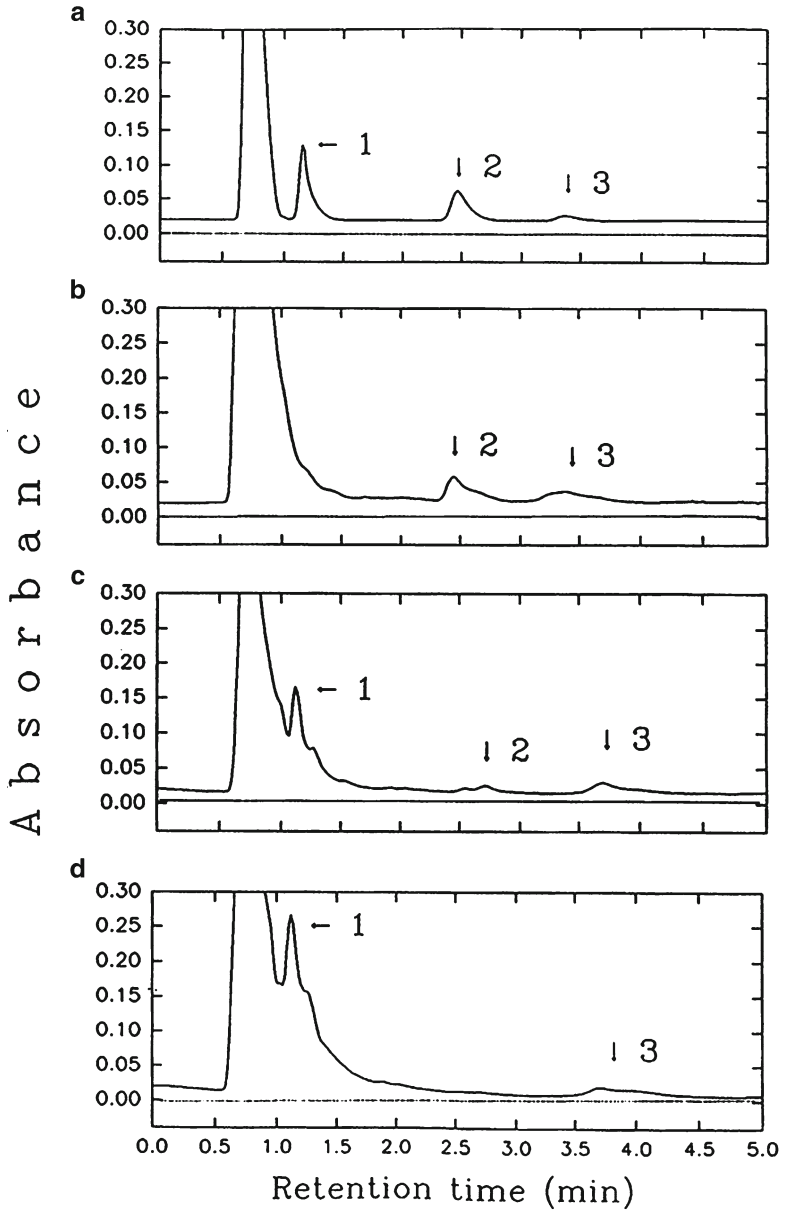

Fig. 7.5 Representative HPLC recordings of cholesterol species in the cardiomyocytes ± cholesterol oxidase treatment. (**a**) Standard cholesterol and oxidized cholesterol species, peak 1:4-cholesten-e-one; peak 2: cholesterol; peak 3: 20 α-OH cholesterol; (**b**) Control cardiomyocytes. (**c**) Cardiomyocytes were incubated with 1 mg/ml CO for 60 min at 37 °C; (**d**) Cardiomyocytes were incubated with 2 mg/ml CO for 60 min at 37 °C. Catalase was present at ratio of 1:1 (mg/ml) with CO in (**c**) and (**d**)

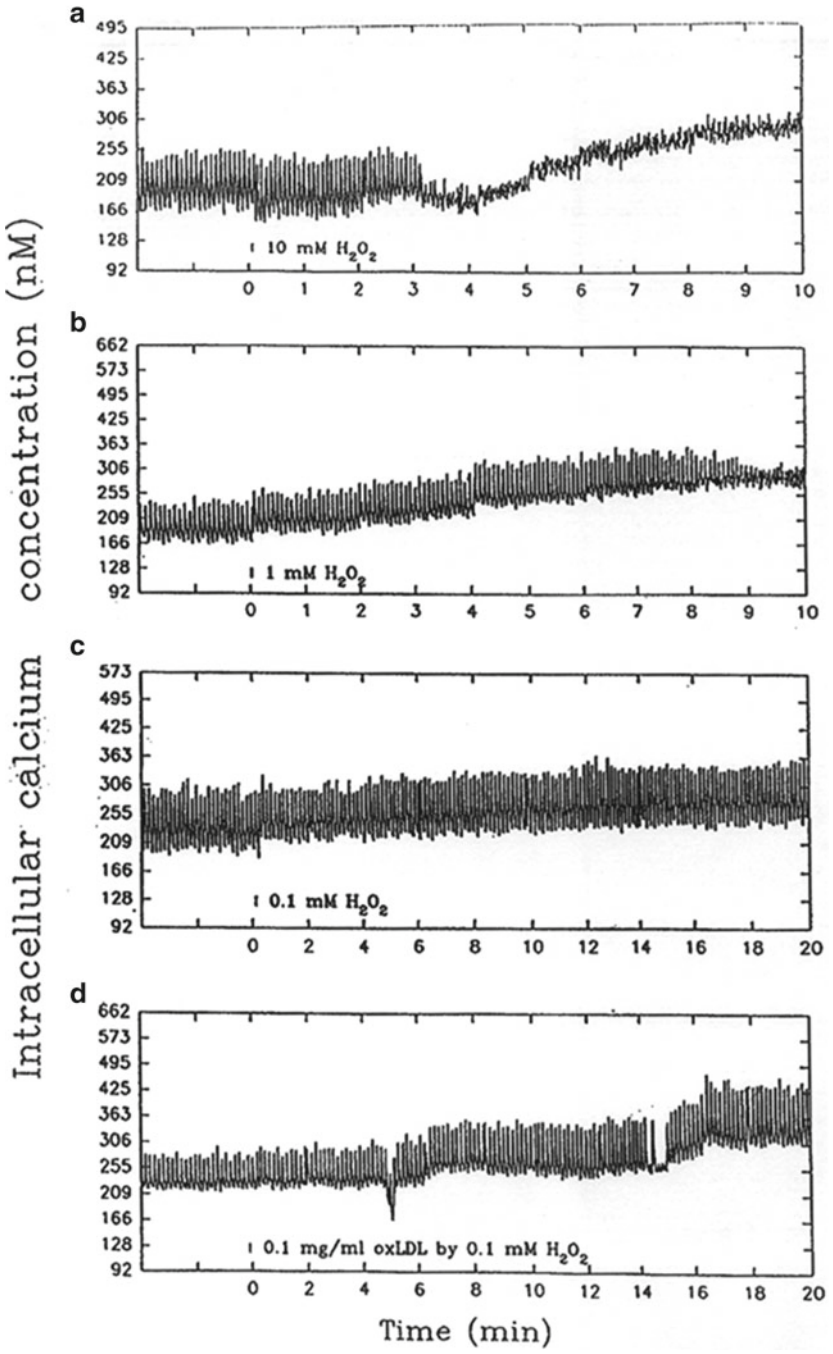

Fig. 7.6 Representative recordings of calcium transients in myocytes after exposure to 10 mM H_2O_2 (**a**), 1 mM H_2O_2 (**b**), 0.1 mM H_2O_2 (**c**) or 0.1 mg/ml oxLDL oxidized by 0.1 mM H_2O_2 (**d**). Note the difference in the time to a change in diastolic [Ca^{2+}] after addition of various concentrations of H_2O_2 (**a**–**c**) and the increment in the calcium transient after cells were treated with 0.1 mg/ml LDL oxidized by 0.1 mM H_2O_2 (**d**)

(Fig. 7.6a–c). 10 mM H_2O_2 treatment produced a rapid (4 ± 1 min, n = 4) increment in the diastolic [Ca^{2+}] of treated cardiomyocytes (Fig. 7.6a). 1 mM and 0.1 mM H_2O_2 also produced an increment in diastolic [Ca^{2+}] in treated cardiomyocytes but it required a longer time to develop (10 ± 2 and 28 ± 2 min, respectively, n = 4–6) (Fig. 7.6b, c). However, the effects of H_2O_2 on the cell Ca^{2+} transient were very different if LDL was present. If LDL was preincubated with 0.1 mM H_2O_2 and then this oxLDL was incubated with cardiomyocytes, an increment (54.4 ± 4 %) of the systolic Ca^{2+} transient of cardiomyocytes was observed (Fig. 7.6d) (n = 4).

The time to contracture of cardiomyocytes after treatment with varying concentrations of H_2O_2 was also measured. The cells first lost excitability and then suddenly shortened to a rounded state indicative of contracture. A very rapid contracture (2.4 ± 0.3 min) of cardiomyocytes was produced by 20 mM H_2O_2 treatment (Fig. 7.7). When the concentration of H_2O_2 was decreased to 0.1 mM, a much longer time was required for cardiomyocytes to develop a contracture (28 ± 2 min).

7.4 Discussion

The treatment of LDL by CO alone will oxidize LDL cholesterol and generate H_2O_2, which in turn can oxidize lipids in the LDL moiety [29, 43]. By including catalase in the incubation of LDL with CO, we can negate the effects of H_2O_2 and separately define the roles of cholesterol oxidation and fatty acyl chain peroxidation in the effects of oxLDL on Ca^{2+} transients. Several lines of evidence suggest that it is fatty acyl chain peroxidation rather than oxidized cholesterol in the LDL moiety which plays the primary role in the oxLDL-induced alteration of Ca^{2+} transients. First, a good correlation was observed between the MDA content in the oxLDL and the in-crease of systolic [Ca^{2+}] in the treated cells. MDA is an indicator of lipid peroxidation [2, 19]. Secondly, catalase effectively protected LDL from lipid peroxidation and also protected against the change in Ca^{2+} transients in the treated cells. This would further support the conclusion that an interaction of the lipid peroxidation products in the oxLDL with cardiomyocytes may play an important role in modifying the Ca^{2+} transient. The protective effect of catalase against the MDA production in the oxLDL was more efficient than that of lazaroid and vitamin E. This would suggest that LDL peroxidation was primarily induced by H_2O_2 since catalase is a more efficient chelator of H_2O_2 [9, 39]. Lazaroid and vitamin E are good general free radical scavengers [8, 12, 35, 36, 45]. The peroxidation of LDL lipids by H_2O_2 may also have been potentiated by presence of μM Fe^{2+}. Lazaroid has some metal chelating antioxidant properties [5] and its ability to inhibit MDA formation would give an indication that Fe^{2+} may have had a small contributory role in the H_2O_2 effect. Third, H_2O_2 treatment of LDL induced the same increase in systolic [Ca^{2+}] in the cell that CO treatment of LDL (– catalase) did (Fig. 7.5d). Together, these data strongly suggest that H_2O_2 induced fatty acyl chain peroxidation in the LDL and it was these products which caused the change in systolic Ca^{2+} levels. Conversely, when LDL cholesterol was selectively oxidized by CO in the presence

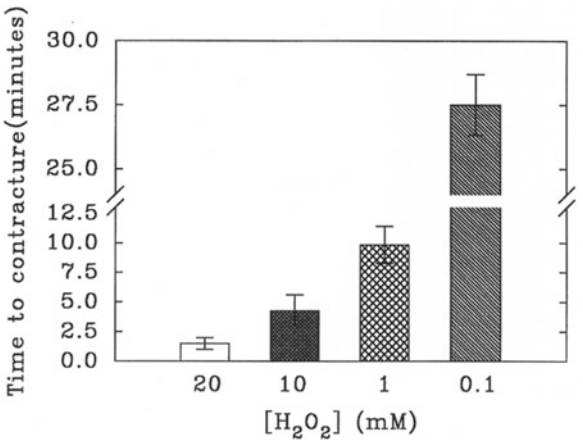

Fig. 7.7 Average time to contracture in myocytes exposed to varying H_2O_2 treatments. Values represent mean ± S.E.M. of 4–6 separate cell measurements

of catalase, no change in systolic [Ca^{2+}] was observed. This clearly rules out oxidized LDL cholesterol as a mechanistic factor.

The effects of CO treated LDL on the cell [Ca^{2+}] were very different than the effects of CO or H_2O_2 in the absence of LDL. Exposure of cells to CO without LDL produced a loss of excitability, an increase in diastolic [Ca^{2+}] and eventually cell contracture. Systolic [Ca^{2+}] was not increased as was the case when cells were exposed to oxLDL. It is very likely that oxidation of cell membrane cholesterol by CO produced nonspecific membrane damage resulting in a leakage of Ca^{2+} into the cell. HPLC data showed the generation of 4-cholesten-3-one in the membrane with a corresponding decrement of membrane cholesterol after the cardiomyocytes were treated with CO. In isolated cardiac sarcolemmal membranes, oxidation of membrane cholesterol resulted in significant changes in the Ca^{2+} flux and an increase in passive Ca^{2+} permeability characteristics [24]. Catalase, the specific chelator of H_2O_2, did not provide any protection against the effects of CO on cell contracture.

At higher concentrations, H_2O_2 also has its own effect on the intracellular diastolic Ca^{2+} level and can cause cardiomyocyte contracture. Our results agree with other studies that this effect of H_2O_2 is time and concentration dependent [16, 20]. There are several mechanisms for the increase in the diastolic [Ca^{2+}] caused by H_2O_2. These include: (1) an inhibition of sarcolemmal Na^+-K^+ ATPase, resulting in intracellular Ca^{2+} overload via Na^+-Ca^{2+} exchange [23]; (2) nonspecific membrane damage caused by sarcolemmal lipid peroxidation which renders the sarcolemma leaky to Ca^{2+} [44]; (3) H_2O_2 might depress the sarcoplasmic reticulum Ca^{2+} pump function which may lead to Ca^{2+} overload in the treated cardiomyocytes; (4) accelerated calcium influx through voltage gated calcium channels [20, 27].

The findings of the current study have significant pathological implications for the heart. Cardiac dysfunction and damage produced by oxygen derived free radicals may be important in ischemia/reperfusion or hypoxia/reoxygenation conditions [7, 33]. Chemical, biological or radiological challenges from the environment

due to pollution, accidents or warfare may also increase the generation of oxygen-derived free radicals in the body and ultimately lead to the cardiac dysfunction and damage shown in the present work. Previous work has demonstrated that LDL can potentiate the effects on Ca^{2+} transients of oxygen derived free radicals on their own [27]. Lipid peroxidation is thought to play a critical role in in vivo situations where oxygen radical mediated tissue damage is important [2, 17, 42]. Our study suggests the fatty acyl groups found in LDL phospholipids, triglycerides and cholesteryl esters are the most important component in oxLDL for stimulating the Ca^{2+} transient of isolated cardiomyocytes. Oxidation of LDL free cholesterol does not account for the effect on the Ca^{2+} transient produced by oxLDL. Protection against LDL peroxidation by antioxidants like lazaroid, vitamin E and especially catalase may have important clinical applications. Clearly, in view of the differences we observed in the effects of H_2O_2 on cell function in the absence or presence of LDL, the present data demonstrate the importance of studying free radical effects in a medium which contains circulating lipids. These data provide important mechanistic information to help explain how damage to the heart can be induced when oxygen-derived free radicals are generated under conditions of excessive exposure to chemical, biological and radiological compounds. The data also demonstrate the importance of using antioxidant agents to prevent this damage.

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Chapter 8
Cerium Oxide Nanoparticles Counteract the Oxidative Stress in Cardiac Progenitor Cells

Francesca Pagliari and Paolo Di Nardo

Abstract Cardiac progenitor cells (CPCs) are a promising source of cells for cardiac regenerative medicine. However, the poor results obtained after a decade of intensive investigation have suggested that innovative protocols must be setup to preserve progenitor cell regenerative potential during the expansion procedure in vitro. Indeed, CPC culture in vitro requires the presence of micro-environmental conditions closely mimicking the natural cell surrounding in vivo. The capability of this micro-environment to uphold reactive oxygen species (ROS) within physiological levels in vitro is a major requisite. Cerium oxide nanoparticles (nanoceria) are redox-active and could represent a potent tool to control the oxidative stress in isolated CPCs. The exposure to 5, 10 and 50 μg/mL of nanoceria for 24 h does not affect cell survival and function in cardiac progenitor cells, while being able to protect CPCs from H_2O_2-induced cytotoxicity. All the tested concentrations have been effective in protecting CPCs from the oxidative stress in the long run and no evidence of toxic effects was detectable, indicating that nanoceria is an effective antioxidant. Therefore, these findings confirm the great potential of nanoceria for controlling ROS-induced cell damage.

8.1 Introduction

The implementation of cardiac cell therapy in the clinical setting has shown to be a very complex endeavor for which a completely innovative vision and novel technologies are necessary. Indeed, in spite of intensive scientific and economic efforts,

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the technological exploitation of the stem cell potential has been carried out adopting very simplistic approaches that are unfit to setup clinically safe, reliable and cost-effective procedures. Consistently, a meager number (approx. 3–10 %) of stem cells home after injection into the myocardium independently of the approach adopted (intramyocardial, intracoronary, retrograde coronary venous) with inconsistent improvements in heart function. Most of the injected cells are suppressed by apoptosis or removed by the bloodstream and, then, entrapped into the lungs [1, 2].

The failure of the injected stem cells to repair the damaged myocardium is caused by technical mistakes in, at least, two steps of the cardiac cell therapy process. In fact, the few stem cells isolated from a donor niche are sorted out by the expression of membrane protein complexes representative of the cell phenotype and cultured in vitro to generate thousand cell daughters [3]. Progenitor cells, improperly cultured in conventional culture conditions, are then exposed to enzyme manipulation damaging cell membrane and destroying the self-produced extracellular matrix required for homing and differentiation [4, 5]. In vivo, stem cells are segregated in special regions (niches), where the micro-environment contributes to preserve their peculiar characteristics and from which they migrate and differentiate upon appropriate stimuli to restore the damaged tissue [3, 6]. Therefore, progenitor cell phenotypic integrity and potential can be maintained in vitro only reproducing environmental conditions emulating the native niche [6–8]. In this context, also the delivery system plays a crucial role in progenitor cell engrafting. At present, all trials have used injections to deliver to the myocardium progenitor cells already injured by inappropriate culturing conditions and floating into the medium. Cell injection exposes progenitor cells to additional distressing conditions (such as syringe and intra-needle pressure, and host critical ischemic microenvironment), to which they, usually embedded in the less-stressed atrial or apical myocardium [9], are not structurally and functionally adapted. Taken together, these drawbacks make unpredictable the number and differentiation of the implanted stem cells as well as the graft shape, size and location.

Cell injection limitations could be more efficiently circumvented adopting sophisticated procedures inspired by tissue engineering concepts. It is possible to fabricate ex vivo portions of tissue to be re-implanted into the damaged organs combining cells and scaffolds made of biocompatible materials. This approach requires a multilevel strategy (Fig. 8.1) in which the quanta of knowledge resident in different disciplines are merged with the sole target of fabricating ex vivo spare body parts to be implanted in diseased organs to exclusively substitute its damaged region and function.

8.2 Sorting Out Progenitor Cells for Cardiac Tissue Engineering

Different cell types have been used to repair the myocardial damage owing to the inability of post-natal cardiomyocytes to proliferate [10]. After implantation in canine hearts affected by dilated cardiomyopathy (DCM), skeletal myoblasts [11]

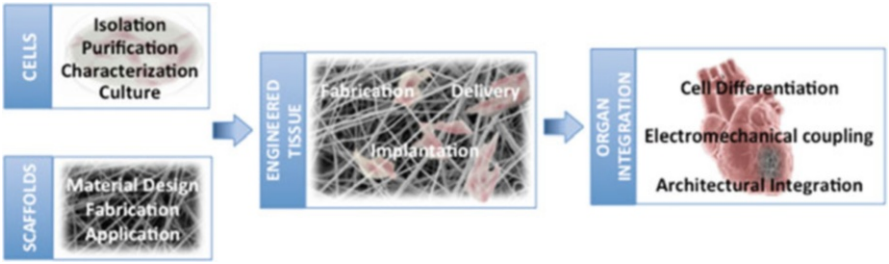

Fig. 8.1 Tissue engineering requires a multilevel approach and strictly standardized procedures in each step to govern stem cell differentiation and integration into the host organ

reduced cardiac remodeling [12], but the effects were more related to their fusion with the surrounding myocardial cells than to direct cell differentiation [13]. Furthermore, skeletal myoblasts did not electrically couple with cells in the host tissue, inducing severe arrhythmic events [14]. The identification of progenitor cells within the tissue of many organs [15], although in a very limited number, fuelled the enthusiasm about the possibility of fabricating engineered cardiac tissues. However, progenitor cells require complex protocols to be expanded and can undergo senescence after a few passages in vitro [16] or face malignant transformation [17, 18]. Additionally, they can suffer from a lot-to-lot variability in quality [19] and the use of animal-derived supplements could be responsible of immune rejection events. Stem cell technology needs that materials and procedures are strictly standardized through a long-term process avoiding that unsuitable options for innovative treatments could be delivered in the clinical setting.

Among others, new cardiomyocytes to be implanted in injured hearts can be easily generated from embryonic stem cells could represent the optimal choice for generating [20]. However, ethical issues, the host immunoreaction, the transfer of potentially noxious agents to the recipient and the potential transformation in neoplastic cells hamper their clinical use [21, 22]. Instead, adult stem cells can be isolated from the same patient to be treated and, then, expanded in culture [15]. In the heart, bone marrow-derived stem cells have been credited to be able to generate new contractile cells, while a paracrine effect on the diseased myocardial tissue is universally recognized [23]. Actually, resident cardiac progenitor cells (CPCs) are leftovers of the embryonic life entrapped in the differentiated mammalian tissue [24, 25], where, in strict combination with supporting cells, they reside within special regions (niches) and remain quiescent for short or long periods of time [5].

CPCs were initially sorted out by the c-kit (CD117) antigen [9], but many laboratories failed to isolate c-kitpos cells and, thus, to confirm initial results. More likely, c-kit only transiently marks cardiac progenitors, which, in rodents [3] and human beings, are more stably identified by the expression of the Stem Cell Antigen 1 (Sca-1), an antigen originally identified in activated lymphocytes [26]. Different investigations have extensively demonstrated murine and human Sca-1pos CPC multipotency and capability to differentiate to cardiomyocytes [27–29] , when properly stimulated. Three classes of factors (physico-chemical, biochemical and mechanostructural)

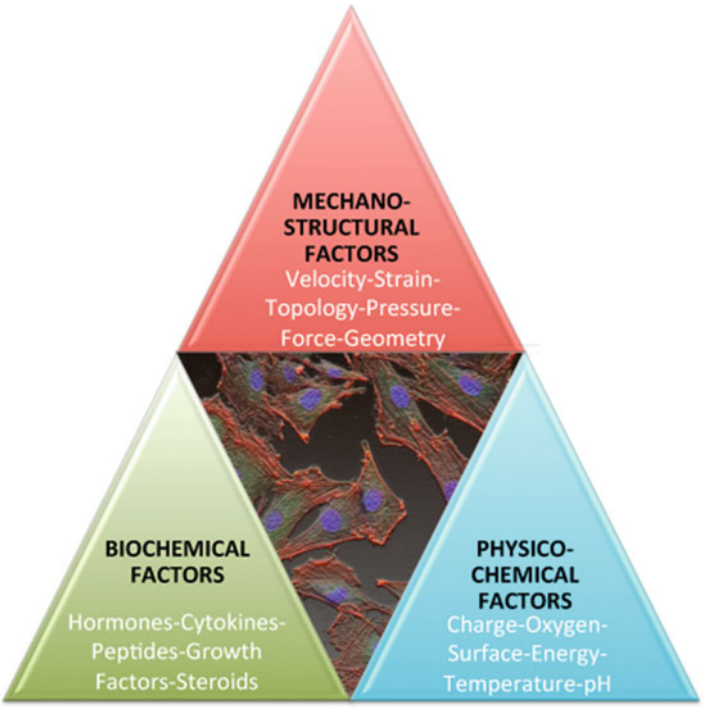

Fig. 8.2 The three classes of stimuli, which dynamically interact to modulate the symmetric environment inducing stem cells differentiation

contribute to determine the stem cell fate [5, 6] (Fig. 8.2) through different levels of equilibrium among them. A pivotal role in this process is played by the mechano-structural signals delivered by the extracellular matrix texture. These signals can be emulated in vitro by properly designed polymeric scaffolds.

8.3 Inherently Bio-active Scaffolds

Scaffolds should display a series of characteristics (Table 8.1) to suitably correspond to clinical requirements and allow the formation of an engineered tissue with predetermined shape. Most of current scaffolds are made of biologically inert biocompatible materials and are intended as mere mechanical support for cells. In order to supply seeded cells with specific biological signals, processes to fabricate smart scaffolds, in which biologically active molecules (growth factors, cytokines, etc.) are embedded into the inert material, are under scrutiny [30–32]. However, this strategy must be changed, since current materials and methods cannot deliver functional heart tissue. Recently, the release of signals sensed as biologically relevant by cells could be possible modifying the chemical structure and topology

Table 8.1 Major scaffold characteristics

Biodegradable and biocompatible
Immunologically inert and atoxic
Enable diffusion of nutrients, oxygen and catabolites
Allow cell attachment, migration, growth, differentiation and vascularization
Electrically intragate with native tissue
Support cell organization and orientation in vivo

of inert scaffolds "Inherently Bio-Active Scaffolds" (IBAS) [6]. In fact, the chemical, physical and mechanical features (stiffness, surface roughness, porosity, micro- and nano-architecture, etc.) of the scaffold can drive stem cell differentiation, even in the absence of specific biological cues [33–35]. Therefore, scaffolds used in tissue engineering cannot be a mere mechanical cell support, but must be able to release signals perceived as biologically relevant by both stem and differentiated cells, thus mimicking in vivo microenvironment [36–38]. Scaffold characteristics are crucial when stem/progenitor cells are used to fabricate architecturally complex engineered tissues, such as the myocardium. Properly combining physico-chemical (polymeric scaffolds) and biological stimuli (neonatal cardiomyocytes) allows emulating an artificial niche able to efficiently drive the cardiac progenitor cell fate [6]. Conversely, embryonic and neonatal cardiomyocytes quickly lose morphological and functional properties when cultured on rigid matrices [39, 40]. Therefore, the design of biologically active scaffolds is dependent on the fundamental understanding of how cells coordinate their functions in the in-vivo environment and in engineered matrices. Therefore matrix/cell and scaffold/cell interactions must first be properly investigated through both numerical modeling and experimental assessment, and only then they can be validated in vitro and ultimately in vivo.

8.4 Strategies to Fabricate Cardiac Engineered Tissues

Differentiated and non-differentiated cells and scaffolds with different chemical and design properties are under consideration [41–44] to fabricate engineered heart tissue, but their clinical application is not at hand, yet. In fact, some protocol steps remain to be defined. First, cells culture conditions remain to be standardized. Second, the target tissue in the host heart is subjected to inflammatory processes of different severity and no specific investigation has been so far designed to define the optimal implantation timing nor to develop procedures specifically designed to prepare the host tissue to receive the implant. Finally, investigators' attention has been focused on fabricating heart portions made of a single cell type, i.e. cardiomyocytes or stem cells supposed to differentiate to cardiomyocytes, neglecting the cell type multiplicity and architectural complexity of the heart. Indeed, cardiomyocytes, representing only 10–20 % of cells in healthy human hearts [45, 46], could be

not sufficient to efficiently repair the texture of the myocardial tissue, which also includes fibroblasts, smooth muscle cells, endothelial cells and adipocytes, among others. Current protocols for heart repair assumed that cell therapy of injured hearts can be limited to restoring the original number of the working cardiomyocytes, only. Results so far obtained have shown that this approach does not re-establish the organ integrity and induce only modest heart structural and functional improvements mostly due to paracrine factors rather than to the mechanical support granted by newly implanted cells [47]. However, very likely, the re-establishment of the original cardiomyocyte number is not necessary to maintain the functional efficiency in the injured hearts. Patients with subcritical necrotic regions subsequent to heart infarction and adequate ejection fraction (>40) can survive without major restrictions to their daily activities. Therefore, cardiac cell therapy should aim at preserving the number of contractile myocardial cells and the organ performance above a critical threshold without necessarily substituting all the damaged cells. To this purpose, the implanted cells must be induced to differentiate to cardiomyocytes and other cell types to be architecturally integrated in different cardiac structures (muscle, vessels) suffering from local (infarction) as well as widespread (cardiomyopathy) tissue damage. Among others, pillars of this new vision are the capability to design a novel generation of scaffolds mimicking the texture of the healthy myocardium and to devise more efficient protocols to culture cardiogenic progenitor cells.

8.5 Implementation of Novel Protocols for Heart Repair

The ideal materials and architecture to fabricate scaffolds for cardiac tissue engineering are still unknown and many controversial issues regarding cardiac stem cells characteristics, and their isolation, identification and expansion remain to be addressed. Indeed, no material in the human body is monocomponent and all senses – be they chemical or physical – are integrated, processed and fuzzy. The complexity must be challenged and met exploiting the plethora of knowledge acquired in chemistry and physics to create new hybrid composite biomaterials and alternative transduction modalities. New approaches should consider the mechanical, chemical and topological signals and design new complex multi-shell or nanocomposite materials able to encapsulate cells, which not only allow induction or control of cell fate but also enable dynamic remodeling of the ECM. Using intuitive and adaptive complex materials, different microsystems can be assembled into a modular engineered tissue.

Over the last few years, a new generation of matrices combining the advantageous properties of natural and/or synthetic materials with nano-components has been proposed for tissue and organs replacement. Nanotechnology involves the manipulation of materials manufactured with dimensions ranging from about 1 to 100 nm and showing novel and unknown properties and functions at nanoscale level. Currently, nanometer materials are widely used in several industrial applications, such as electronics, information technology and aerospatial engineering, but, more

recently, they are also being increasingly used in biomedical diagnostics and drug delivery [48, 49]. Moreover, DNA fragments-carrying nanoparticles are investigated as possible strategy for gene therapy and iPS production [50].

The application of nanotechnology to regenerative medicine has made significant advances enabling the development of nanostructured biomaterials, in order to improve mechanical properties of polymeric substrates and induce specific cell responses [51]. Nano-materials represent a new class of promising materials, since they seem to show chemical, physical and biological properties totally different from their bigger counterpart, thus opening new and exciting perspectives in the multidisciplinary field of tissue engineering [52]. Indeed, the in vivo nano-structured extracellular matrix provides anchorage sites and instructive signals to guide cell behavior, so the ability to control the properties of materials at the nanoscale level could affect the adhesion, proliferation and cell differentiation [53] and help to drive the proto-tissue generation both in vitro and in vivo.

In particular, scaffolds fabricated by incorporating nanofillers into biocompatible/erodible polymeric matrices have gained rising attention to fine-tune materials properties and to meet a broad range of applications. Nanoparticles offer the possibility to design hybrid systems with organic and inorganic characteristics, which exhibit better mechanical and physico-chemical properties than individual components [31]. Among others, cerium oxide nanoparticles are promising nanoparticles displaying the ability to mitigate oxidative stress [54, 55].

Cerium is a rare earth element of the lanthanide series of the periodic table. Its oxide (CeO_2, ceria) has been widely used in various applications, such as oxygen sensors [56], electrolytes for solid oxide fuel cells [57] and ultraviolet absorbent [58]. Although nanoparticles have been extensively studied for their cytotoxic effects on various cell types [59, 60], a few studies have been performed about their potential biological activity. Lately, CeO_2-loaded PLGA scaffolds displaying peculiar mechanical, topographical and biological features have shown to be able to improve CPC adhesion and growth, as compared to matrices consisting of pure PLGA or TiO_2-loaded PLGA, but, above all, to guide cell orientation following the nanoparticles patterns [31]. Such studies also suggested a potential biological role of CeO_2, which could be related to its capability to act as a free-radical scavenger, being able to confer protection to cells from oxidative stress [54]. In this context, the ceria nanoparticles mechanism of action has been related to the presence of a dual oxidation state (Ce^{3+}/Ce^{4+}) on their surface; this implies that the loss of oxygen and the reduction of Ce^{4+} to Ce^{3+} are accompanied by the generation of an oxygen vacancy or defect in its lattice structure [61]. The surface vacancies are believed to be "active sites" [62], where reactions, involving oxidative species, may occur, causing the reversible oxidation of Ce^{3+} sites to more stable Ce^{4+} state. In addition, as particle size decreases, nanoparticles exhibit more oxygen vacancies and Ce^{3+}/Ce^{4+} ratio increases [63]. Recent studies suggested that the potent antioxidant activity of CeO_2 nanoparticles may be related to an auto-catalytic behavior that would result in an autoregenerative reaction cycle by the following mechanism: $CeO_2^{4+} + O_2^{\bullet} \leftrightarrow CeO_2^{3+} + O_2$ [31, 64]. As a direct consequence, CeO_2 can act more efficiently than natural compounds in biological systems. In fact, while natural

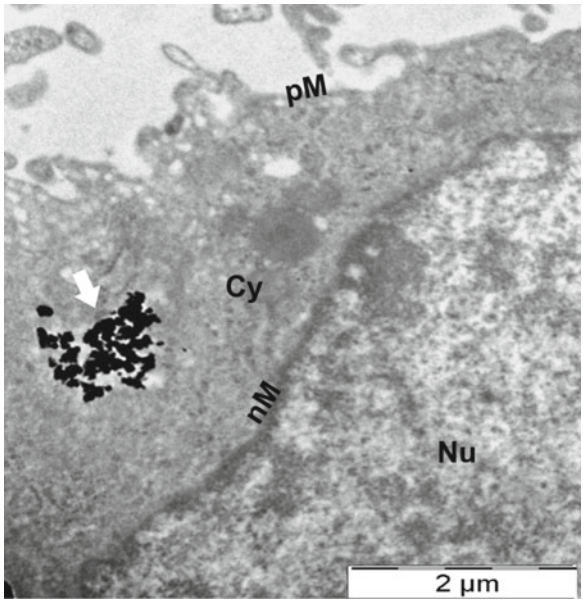

Fig. 8.3 Sca-1pos CPCs displaying clusters of CeO_2 nanoparticles (*white arrow*) into the cytoplasm, 7 days after ceria treatment (50 µg/mL). TEM micrograph. *pM* plasma membrane, *nM* nuclear membrane, *Cy* cytoplasm, *Nu* nucleus

anti-oxidant compounds display one or limited catalytic sites and short half-life, ceria nanoparticles display many active sites for free-radical scavenging, owing to the high surface-to-volume ratio, and catalytic action recovery [54]. In this capacity, CeO_2 nanoparticles have shown the ability to confer radioprotection to breast cells [65] and lung fibroblasts [66] and to act as antioxidant in neuronal cells [61, 64], retinal cells [54], murine macrophages [67], gastrointestinal epithelium cells [68] and human leucocytes [69]. Interestingly, CeO_2 could act by mimicking superoxide dismutase (SOD) and catalase activities [70–72]. The anti-oxidant beneficial effects CeO_2 have been also demonstrated in a model of murine cardiomyopathy [73] and in human cardiac progenitor cells (hCPCs) [55]. In the latter case, hCPCs pretreated for 24 h with three different CeO_2 concentrations (10, 25 and 50 µg/mL) were able to up-take nanoparticles and retain them up to 7 days inside the cytoplasm as aggregated bodies not enclosed by vesicular membranes (Fig. 8.3). Such a treatment apparently did not provoke structural or functional damages, as was apparent from the immunofluorescence micrographs. Moreover, when cells were exposed to an oxidant H_2O_2-mediated insult, 24 h-pretreatment with 50 µg/mL ceria nanoparticles caused a 30 % ROS decrease as compared to untreated hCPCs. The ROS decrease was even larger at 3 and 7 days (from about 50 % up to 75 %) after a single ceria pulse. Lower concentrations of ceria (10 and 25 µg/mL) did not appear to exert any protective effect after 24 h from initial stimulation, but were consistent at 7 days, thus suggesting a longer dose-related activation time to express their antioxidant ability [55]. Altogether, these results clearly demonstrated that the internalized ceria nanoparticles, being able to be active at least up to 7 days, could act as antioxidant agent preserving the integrity of the cellular DNA repair system [74] and the intracellular signaling [75].

8.6 Conclusion

These findings demonstrate the great potential the use of nanoceria could have in the regenerative medicine. In fact, CeO_2 nanoparticles embedded into a 3D scaffold could provide cells with appropriate physical and topographic signals at nanometer scale, similarly to those arising from the extracellular matrix (ECM) [76] in order to address cell fate. On the other hand, CeO_2 antioxidant effects could contribute to create an artificial microenvironment in order to maintain progenitor cells under optimal conditions, during the generation of proto-tissues in vitro.

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Chapter 9
Clinical Trial Complexity Measure – Balancing Constraints to Achieve Quality

Susan Devine, Tammy Mah-Fraser, Dawn Borgerson, Amanda Galster, Susan Stork, Tina Bocking, Nita Takeuchi, and Kay Friel

Abstract With limited clinical research funding, it is imperative that costs associated with clinical research studies be transparent and measureable to ensure optimal use. The Pediatric Ontario (Oncology) Protocol Assessment Level (Peds-OPAL) provides a mechanism to assess research coordinator workload, a significant component of a clinical research study budget by measuring study complexity. Reflecting research practices congruent with the International Conference on Harmonization Good Clinical Practices, the Peds-OPAL scoring instrument affords investigators and project managers the ability to assess impact on trial quality in relation to constraints of time, cost and scope.

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Fig. 9.1 Project management constraint model

9.1 Project Management – Constraints in Clinical Trials Research

Limited clinical research funding from industry, government, hospitals, philanthropy and other agencies, along with ever increasing scrutiny of research practices, demands that costs incurred within the clinical research process be realistic, transparent and measurable ensuring optimal use and protection of available resources. Associating a clinical trial with the project management model of constraint (see Fig. 9.1) allows us to understand the importance of having compatible scope, time and cost in order to ensure quality [1]. More simply stated, quality (defined as the attainment of study objectives while protecting patient safety) requires balancing the constraints of Scope (study aims), Time (period in which to complete the trial) and Cost (study budget, human resources) and are imperative in today's research environment.

9.2 Research Coordinator Role and Workload Assessment

Research Coordinators, also known as Research Nurses, Coordinators, Clinical Research Professionals and/or Project Coordinators, account for the majority of staff time on a clinical trial project [2]. Two investigational new agent drug studies conducted by the National Cancer Institute of Cancer Clinical Trials Group showed that research coordinator costs represent 21% and 13% respectively [3]; not an insignificant portion of the budget for a clinical research project.

Importantly, having a dedicated clinical research coordinator responsible for the management, administration and data collection involved in a research study is essential to the success of a clinical study [4]. A Research Coordinator contributes to study recruitment and continued participation in clinical trials and is found to directly contribute to good data collection [5]. At the 1999 Annual Meeting of the American Society of Clinical Oncology (ASCO), the then-president Allen S. Lichter reported that in a survey of oncologists, 38.4% responded that insufficient study management support constituted a significant barrier to their participation, and more than 80% said that more coordinator help would assist their enrollment efforts [6].

More research coordination time is needed for early phase studies where patients are seen more frequently, reporting required in real time and regulatory constraints more demanding than for those in later phases. External sponsorship also has an increased effect on coordinator time with increases in areas such as regulatory documentation and on site monitoring [7].

The most common method to measure workload in clinical trials has been to measure or count the frequency and time to accomplish tasks [2, 8–12]. The European Organization for Research and Treatment of Cancer (EORTC) created a workload measurement instrument (WMI) to assist centers to accurately record time spent on different activities which could be used to calculate the real cost of conducting the oncology trial [13]. The instrument has been validated and completed [14].

Ontario Institute for Cancer Research (OICR) developed the Ontario Protocol Assessment Level (OPAL) a protocol workload assessment of clinical trial protocols. The guiding principle for this instrument was that it be an easy to apply scale that measured work tasks specific to the research coordinator and included all trial phases [15]. Rather than logging hours and tasks, this group developed a protocol rating scale based on clinical trial complexity. The OPAL scale, when used in combination with the number of subjects recruited to each clinical trial, allows coordinators to measure their personal workload and produce a score.

9.3 Peds OPAL (Pediatric Ontario [Oncology] Protocol Assessment Level

Following the development of OPAL, a pediatric study committee, having access to more than 200 sites running the same clinical trials, initiated a randomized concordance study using a pediatric version of the OPAL instrument called Peds OPAL. This study has been completed and results are pending submission for publication.

Peds OPAL is reflective of the adult OPAL instrument created by OICR with minor alterations to reflect differences in the pediatric cancer clinical trial setting. It is an effective approach to measuring workload as it is simple to apply, and while it can be reflective of individual research coordinator workload, the pediatric group preferred to simplify workload scoring further by assessing workload as a site score. The availability of a site score could be of benefit to sponsors and funders when assessing potential research sites.

The Peds OPAL instrument (Fig. 9.2) base scores, 1 through 8, assume all core tasks that cross all clinical research studies such as informed consent of human subjects. Additionally the base score incorporates Phase specific core tasks in its basic assumption such as adverse events in all phases of therapeutic trials and pharmacokinetic studies in Phase I trials.

Tasks that increase workload are categorized as *special procedures* which are done for research purposes only and might include items such as bone marrow transplant, special laboratory tests or multiple randomizations.

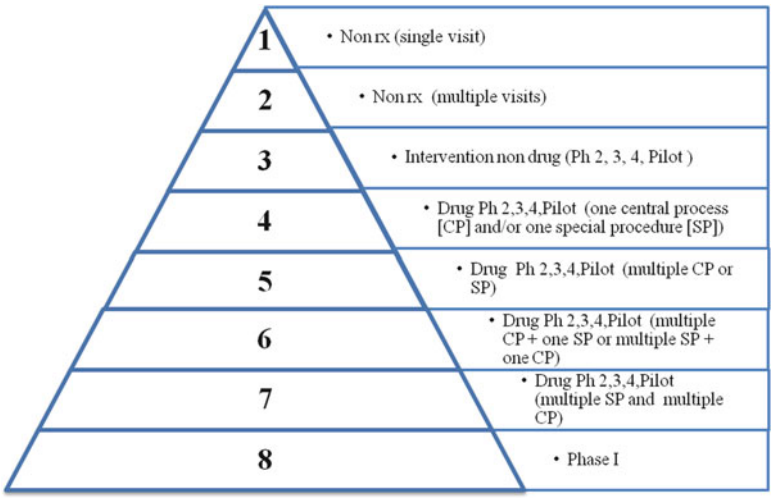

Fig. 9.2 Peds OPAL protocol complexity instrument

A second category of increased workload is *central processes* defined as the submission of materials for an external review where results are returned for clinical purposes or a study mandated secondary review. This category could include tasks associated with external review of pathologic diagnosis, quality assurance of radiation therapy treatment or a confirmation of response by a central review of imaging.

Every special procedure and central process involves increased time spent in study and patient coordination, planning, reporting as well as regulatory compliance; all of which impact research coordinator workload and therefore increases the Peds OPAL complexity score.

Although the base score incorporates core tasks common to all clinical research, and increases with the addition of special procedures and central processes, there are some additional elements that require inclusion. These additional or bonus points labeled '*Add Ons*' are a unique set determined for the pediatric cancer population. Fig. 9.3 is a comparison of the *Add Ons* for the pediatric versus adult cancer populations; the only component not identical in OPAL and Peds OPAL.

9.4 Balancing for Quality

In project management terms, a clinical trial fits the definition of a project; a temporary endeavor performed to produce a result [1]. There are three main components that define a project: *Scope*, desired results of the project; *Time*, timeline from start to end of the project; and Required *Resources*, people, money and equipment necessary to complete the project. Each component affects the other two; thus increasing study objectives or scope will affect how long it takes to complete a project as well as

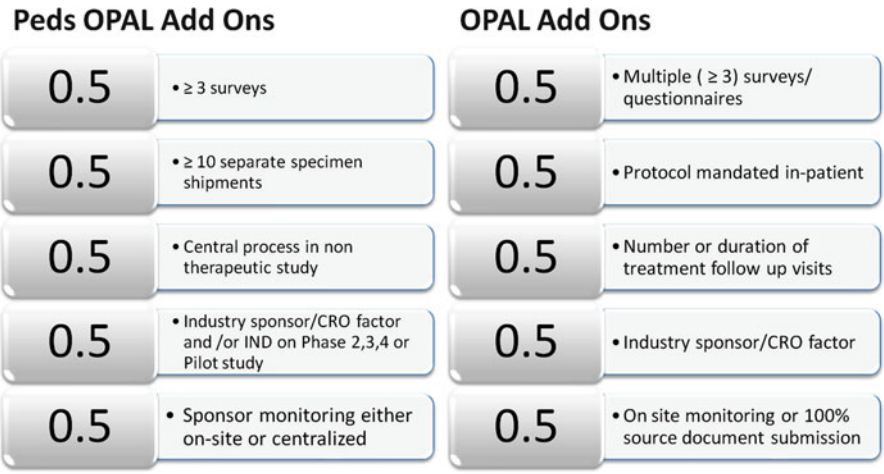

Fig. 9.3 Peds OPAL and OPAL *Add Ons* comparison

Fig. 9.4 Constraint model in clinical trials

the resources, both human and financial required. Using PedsOPAL to measure coordinator workload, provides an opportunity to carefully and correctly assess resources for a Research Coordinator; an important member of the research team and significant part of the study budget.

9.5 Conclusion

Quality in clinical research studies requires both patient safety and study data integrity delivered within an agreed upon budget. Research Coordinator workload is a necessary and significant cost in clinical research budgets. Therefore, the scope of every clinical research study should include attainable, measurable and important objectives cognizant that for every objective there is time and cost associated. Understanding how constraints of scope, time and cost affect each

other is important to maintaining quality. The Peds-OPAL protocol complexity scale assists by providing an objective measure of Research Coordinator required effort.

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Part III
Bioactive Compounds from Natural Sources for Prophylaxis and Treatment of the Effects of Radiological, Chemical and Biological Agents

Chapter 10
Systemic Approach in Determining the Role of Bioactive Compounds

Alexandru Dascaliuc, Raisa Ivanova, and Gheorghe Arpentin

Abstract Biosystems theory provides a useful framework for describing the biological role of bioactive compounds. Each functioning biosystem must adapt to and, to a certain degree, possess mechanisms or means to control both intra- and extra-organism conditions, "*purpose*". The organism must have the capabilities to change itself or the external environment, including community function, to achieve a purpose at the highest possible level. In general under adverse conditions, an organism is forced to renounce the purposes of higher level and to fulfill the purposes of a lower level. The achievement of higher level purposes depends on the production of natural bioactive compounds (adaptogens). The role of bioactive compounds is discussed herein from the point of general concept of biosystems, their resistance, and their adaptation to adverse conditions. Utilization of the concept of a biosystem will provide a clearer understanding of the role of bioactive compounds in the sustenance of an individual redox state and to predict new functions and properties of the organisms and their bioactive compounds.

10.1 Introduction

For many years, humankind has benefited from the use of green plants and animal products as sources of drugs and herbal remedies. Natural drugs (e.g., antibiotics) derived from microorganisms have a much shorter history of exploitation to improve human health. Their major impact on medicine goes back only about 60 years to the

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introduction of the antibiotic penicillin to control communicable diseases. Microbial produced antibiotics now account for a very high proportion of the drugs commonly prescribed. Animal related drugs, such as steroids and prostanoids, are also in large usage. With time, the concept that the exact boundary between remedies and food products does not exist is emerging. One of the confirmations of this observation is the recognition that some products, for example from fungi, concomitantly represent pharmaceuticals and health foods. It is also well known that the quality of plants, animal, and microbes as source of medicine and food depend on conditions of habitat or cultivation.

Two primary areas of interest that are emerging relating to these biological products are a elucidation of their role in the general metabolism of the individual organism and the importance of these substances to the functioning of the biological community considered as a whole. These studies have led to the development of a general theory of biosystems development and their environmental interactions, which is outlined in the current article.

10.2 Concept of Biosystem: The Basic Properties of Biosystems

Each biological object represents a biosystem in itself in that the intracellular organization of less complex organisms as well as the complex of organs comprising higher organisms necessitates specific controlled interactions of the organisms' components [1]. To simplify our discussion, the levels of biosystems could be divided into three categories: the *under-organism level* (intra-organism interactions) – cells, tissues, organs; *the organism itself*, and the *super-organism level* – (inter-organism interactions) – population, ecosystems, and biosphere. It is clear that each biological entity is a concomitant element of the next higher level system as well being a system unto itself *per se*. The higher level systems integrate all of the processes of the lower level systems contained therein into a communal whole; directly and indirectly, regulating or influencing all inferior lower levels of integration [2]. A necessary consequence of this multilevel interaction, that with each new level of structural and functional interaction, new functions must appear, which are frequently impossible to be predicted based on simple observations of the activity of the various lower-level components. As a result, when analyzing the properties of a biosystem, it is necessary to explore not only the links at the same level (e.g. *processes of development with time*), but also the vertical interdependence of phenomenon at different levels of integration.

A primary concept essential for understanding the function of biosystems is the fact that they are open systems [3]. The existence of this open system impact nature and quantity of its products as well as various aspects of energy assimilation, accumulation, transmission, and utilization, all associated with ensuring maintenance of the biosystem structure, its growth and function. *The second* side of function is related to orientation of energetically processes. It includes perception, storage,

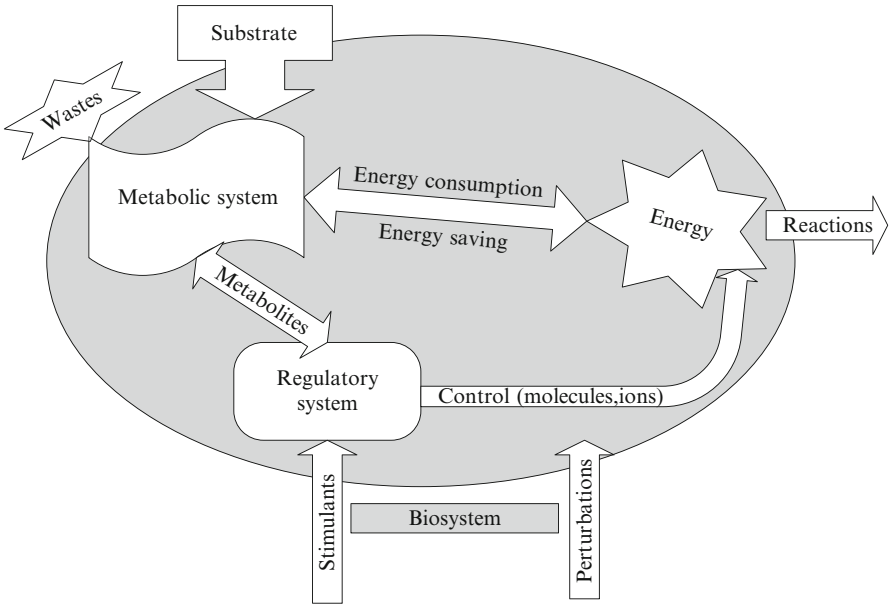

Fig. 10.1 Substrates, energy, and information flow in biosystems. See details in text

processing, and utilization of information. The mechanisms that handle information determine the nature of active energetic process and their rates in the system. This aspect of function is responsible for determination of the rate and direction of biosystem development and adaptation.

A depiction of energy and information flow in relation to system function is provided in Fig. 10.1. The energetic component is designation as *metabolic system* (***MS***); the operational portion of the process is presented from the view of regulatory system (***RS*** – *genetic and physiological regulation*), and blocks of the effectors. In this schematic, two the main characteristics of biosystems are demonstrated – nutrient exchange (*the opened portion of the biosystem*) and process control.

The anticipated final state of a biosystem, which is primarily the product of its function or a final structural status achieved in consequence of its physical organization, has been termed the "*the purpose mechanisms of handle*". In accordance with the theme of the workshop, we discuss only the components of "*purposes*" that ensure life through provision of all necessary metabolic and energy resources (agents) and with protection of life functions from the damaging factors of the organism's environment. *The first aspect* of *maintenance capacities* of biosystems is related to insurance of constant internal conditions necessary for sustenance of "life", *homeostasis*. *The second aspect* of maintenance of biosystems derives from the necessity to provide adequate acquisition of nutrient and energy resources from sites outside of the organism itself. This assures the *stationary non-equilibrium state*. The stationary non-equilibrium status between processes of input and output

of substances and energy is characteristic for all living systems, but only under conditions of homeostasis are they at optimal (economical) levels.

Sustainability of biosystems can conveniently be discussed in the terms of *flow rate* and *levels* [4]. When describing living systems, we are operating at two levels. *Firstly*, the content of energy and metabolic resources within and outside the biosystem must be considered. The variables to be considered in this aspect are termed, *levels*. *A second level* of consideration involves evaluation of the flux rates of these substances, such as the dynamics of accumulation, uptake, assimilation, recycling and excretion of substances. These are designated as process *rates* – rates of enzyme synthesis, rates of growth, rates of oxygen utilization etc. The achievement of a reasonably constant internal environment requires maintenance of the level of nutrient resources and energy in the interior of the system. Biological regulatory systems, essential in maintenance of the internal and external homeostatic conditions, are in generally directed to achievement of specific essential cellular or organismal functions – i.e., to maintain a *highly efficient level of cellular function* – effective energy and nutrient consumption, economical or efficient utilization of nutrients, and reliable or sustainable function [5].

In Fig. 10.1, the relationships are depicted as *metabolic system, regulatory system*, and *energy*, associated with intra-system level functions. External components are influenced by the intra-system reactions and as well by the intra system activity due existence of signals from interior of this system (waste, reactions, redox state). In biosystems with different levels of organization (microorganisms, higher organisms, populations, ecosystems, and biosphere) external and internal components are of varying complexity. For example, in an RS within the organism activity results from individual genetic capability, but in the community – it necessarily is the product of the genetic capability of different members of the community. That is, biosystem of higher complexity include external signals of all biosystems of which they are composed.

Under adverse conditions, the biosystem must adapt to its environment, as allowed by the capability contained within its genetic makeup, to modulate internal and external functions and to induce modification of the external environment. For example, exhaustion of nutrient and energy resources must necessarily result in a diminution of the level of function of the biosystem. In physiological terms, initially the system is subjected to the strain induced by overall physical and chemical limitations of the system, but subsequently as required resources are diminished or natural conditions degraded, a situation that could be described as pathology occurs [6]. In contrast to the normal situation, that assure optimal conditions for life, the organism must now expend energy and resources necessary to attempt to restore homeostatic conditions in a "pathological" environment. In Fig. 10.2, a hierarchy of three purposes of biosystem under influence of two factors, described with two variables, V_1 and V_2, is shown schematically. A large range of conditions (S_1) exists where the organismal or cellular regulatory mechanisms are capable of assuring a reasonably constant range (*the regime of stationary non-equilibrium state*) of biosystem function. The area (S_2) of more favorable conditions, where the biosystem is capable of maintaining the homeostasis, is more limited. Lastly, we could designate

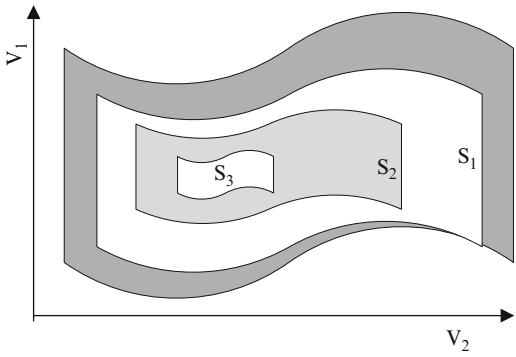

Fig. 10.2 Hierarchy of biosystems' purposes

a more limited range of conditions (S_3) where optimal organismal function occurs. As the most favorable conditions for biosystem functioning are degraded, the parameters of conditions exist outside the narrow region (S_3) and biosystem transitions to a lower level of function (in region S_2). Further degradation of the organism's environment may result in its having to function further from the region S_2 (where biosystem is able to maintain homeostasis). Additionally situations may easily be described where conditions are for region S_1 and even outside this region. Outside the conditions marked by region S_1, the biosystem can only be maintained alive for a limited time period. In Fig. 10.2 this region is designated outside the area S_1. It is important to note that the requirements for conditions of environment that are sufficient for maintaining the biosystem at lower levels of function are necessary, but not sufficient for maintaining them at higher levels. This means that to maintain viability under adverse conditions, the biosystem gradually abandons the higher level purposes and transfers to maintaining the purpose of lower level. Under stressful conditions, the biosystem does strive to achieve the highest possible purpose. When conditions become favorable, the biosystem gradually pass from purpose of lower level to those of higher levels.

From a practical perspective is extremely important to determine the appropriate parameters that indicate the transition of biosystem from one state to another (conditions of stress). This would enable to compare the stress resistance of different biosystems, and as well the influence of various factors on their resistance and as well the recovery of damage caused by stress. An indicator of the biosystem resistance could be the redox state, but it is necessary to consider that in the complex biosystems it can be at different levels in different compartments. As all provoked by stress damages and their recovery may be specifically in biosystem components, processes must be classified in accordance to their levels of manifestation. Figure 10.3 demonstrates the possible ways of recovery of biosystems response to stressful conditions. Under extreme stressful conditions, the response of the biosystem may be the result of many complex interactions, some of which are to distinguish without supplementary experiments. The existence of various mechanisms to withstand stresses reflects the capacity of biosystem to abandon (gradually, in conformity with the degree of aggravation of environmental conditions) the hierarchically less important purposes required for maintaining life.

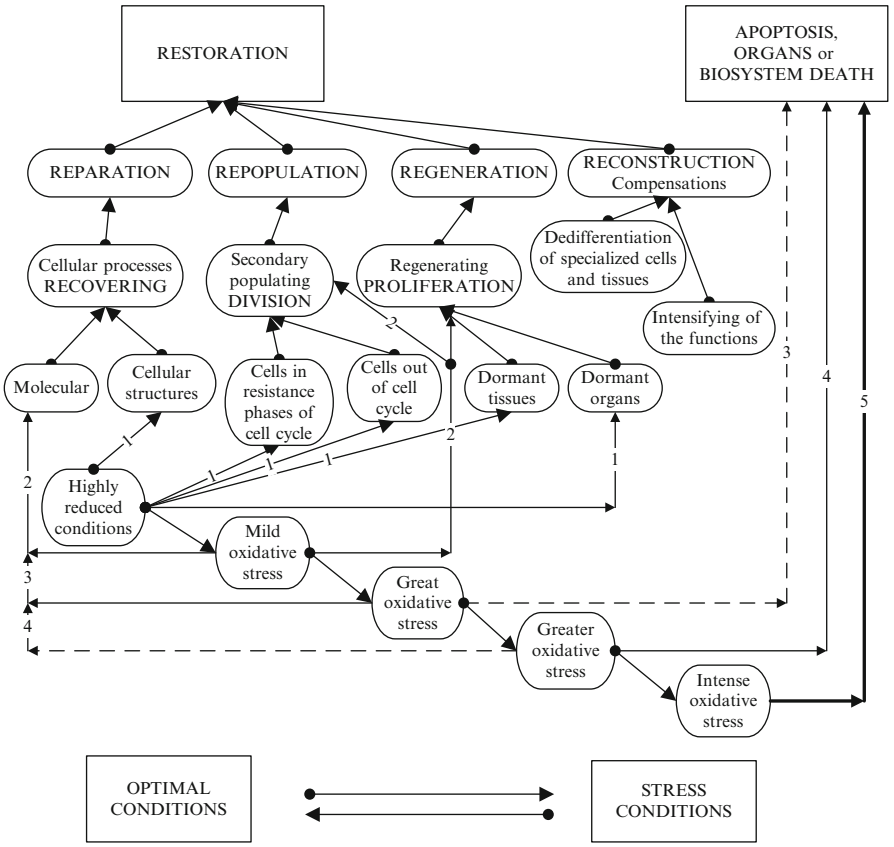

Fig. 10.3 Change of status and possible routes of biosystem recovery in response to stress factors [7]: *1* – highly reduced conditions, *2* – mild oxidative stress conditions, *3* – great oxidative stress, *4* – greater oxidative stress, *5* – intense oxidative stress

Recently much has been made to elucidate the molecular components common for resistance. It was demonstrated that there are several common ways of controlling the plant response to different stressors. The first adjustment is made involving transcription a complex set of interacting signals pathways and provides common and specific induction of resistance. Rate genes encoding proteins involved in biosynthesis of transcription factors is about 25 % [8]. The stress-response relationships illustrated in Fig. 10.3 show the changes in stress conditions of the flow in substrates, energy, and information in biosystems illustrated in Fig. 10.1. The factors that act at early stages of stress response are critical for other biosystems functions. They use common pathways and components in the stress-response relationship. This makes possible cross-tolerance, concomitant adaptation/acclimation to different stresses after exposure to one specific stress. Responses to stressors are based on the general changes that occur under the influence of stress. One of such changes is dehydration. Elements that respond to dehydration and genes that are regulated by

these factors were detected in plants [9]. The activity of transcription factors may be related to the influence of hormones and determined the differences in resistance to stress of related species. A single transcription factor can coordinate the expression of many genes to improve stress tolerance.

It is worth noting that, in many cases, plants survive stress by metabolic arrest, in which growth and development essentially stop. The common response of plants to different abiotic and biotic stresses, such as heat, drought, cold, high-light intensities, wounding, UV, ionising radiation, ozone, and pathogens is the accelerated production of *active oxygen species* (AOS). Among them the most important are hydrogen peroxide, hydroxyl radical, and the superoxide. The action of H_2O_2 as a signal in the induction of different catalase genes has been shown [10]. The peroxidases also control the amount of H_2O_2 present within the plant cell and its concentration reach the level that trigger death only at intense oxidative stress (Fig. 10.3). H_2O_2 is a local and systemic signal in plants adaptation to high light, tolerance to heat shock and low temperatures, growth responses to environmental stimuli [10]. This indicated the important role of the H_2O_2 in induction of the cross cores tolerance and in the stress survival network. Changes in H_2O_2 homeostasis are representing signalling event and induce the enhancement of stress tolerance. When the oxidative stress is intense, high concentration of H_2O_2 may leads to programmed cell death. H_2O_2 interacts with other signalling systems, particularly hormones, modify the action of other secondary messengers such as Ca^{2+} and NO, and act via modification of signal transport system in optimal and stress conditions.

10.3 Bioactive Compounds and Their Role in Biosystem Resistance and Adaptation

Cell processes are regulated also by oxidation and reduction, but phosphorylation and dephosphorylation is equally important. The redox state of the cell influences phosphorylation, and vice versa. Some kinases can be directly and indirectly affected by AOS. Balance between AOS and antioxidants are important. The bioactive compounds (and adaptogens) acts directly as antioxidants, or in conditions of stress have beneficial effects changing the antioxidant – prooxidant balance in favour of the former, leading to potential recuperation. Increased oxidative damage can result not only from more oxidative stress, but also from failure to repair or replace damaged biomolecules.

It is generally concluded that bioactive compounds influence an organism's health and resistance to different adverse environmental stresses. An important step in understanding the nature and mechanisms of their influences on biosystems appeared after introduction in 1947 of the concept of adaptogens by Lazarev et al. [11]. They were defined as substances meant to put the organism into a state of non-specific heightened resistance in order to improve resistance to stresses and to adapt to extraordinary challenges.

As in the case of stress, the conception of adaptogens was initially developed to explain the protective effects of some natural products on human and animal systems and was later extended to include their effects on all biosystems. There are well known parallels between the action of some substances and occurrence of disease resistance in plants and animals. The most impressive example of such substance is salicylic acid (SA) and its derivatives, isolated from extracts of different plants. It appears to have multiples modes of action since exert a wide range of clinical effects including reduction of pain, fever, inflammation, blood clotting, and the risk of heart attacks and strokes. Exogenous supplied SA has been shown to affect a large variety of processes in plants, including stimulation of stoma closure, seed germination, fruit yield and glycolysis. Adaptogens help biosystems to promote the capacity to rapidly adapt at the cellular, tissular and organism level to different stress factors in process termed Non-Specific Resistance Stimulants NSRS (for animals) and Systematic Acquired Resistance SAR (for plants) [12, 13]. The areas of the adaptogens benefits are stress protective action, activation of immune system, improvement of mental and physical work capacity, normalizing effects, antioxidant and anti-aging action, augmentation of performance, endurance and rehabilitation etc.

Unfortunately, one very essential feature of adaptogens is not fully discussed in the scientific and population literature: that is, their effects are realized at very low concentrations. It is known that mechanism of action of adaptogens depends on many factors, including chemical properties of adaptogen, genotype specificity, and age of organism, stage of development and even season of the year [1]. In our opinion, the different effects of bioactive compound on biosystems are a useful consideration from the view of general theory of biosystem, resistance, stress, and adaptations. This type of analysis could be very important because it is necessary to generalize the causes of existence of reduced number of stress reactions to different stress factors of organisms that belong to different kingdoms. Generally speaking, the beneficial effects of adaptogens could be due to enlarging one or concomitantly many areas (S_3, S_2, and S_1) indicated on Fig. 10.2.

Although a detailed analysis of mechanisms of action of adaptogens is beyond the scope of this article, it could be useful to demonstrate the efficacy of utilizing the systemic approaches in consideration of the role of heat-shock proteins (hsp) [14] in adaptation. The synthesis of these proteins, in prokaryotes and eukaryotes, is induced during a stressful event, such as high temperatures. Additionally, many of the "*stress proteins*" play important roles for normal cellular functions under stress-free conditions, especially in periods of development, differentiation and growth (influencing on recovery of molecular and cell structures (Fig. 10.3). The prompt induction of hsp in stressful situations is a vitally necessary protective function for protection of sensitive cell proteins from denaturation, recovery of damaged protein and reparation of cell structures (Fig. 10.3). They also influence RNA and protein-synthesis, temporarily inactivate certain receptors or initiate immune reactions that will influence repopulation, regeneration, and reconstruction (Fig. 10.3). These effects of *hsp* are extending of the physiological adaptation; protect energy resources from depletion, and accelerate the biosynthesis of proteins and nucleic acids.

A specific example of the adaptogen effect is the experimental results from study of a preparation Reglalg (Fig. 10.4). This preparation includes a mixture of

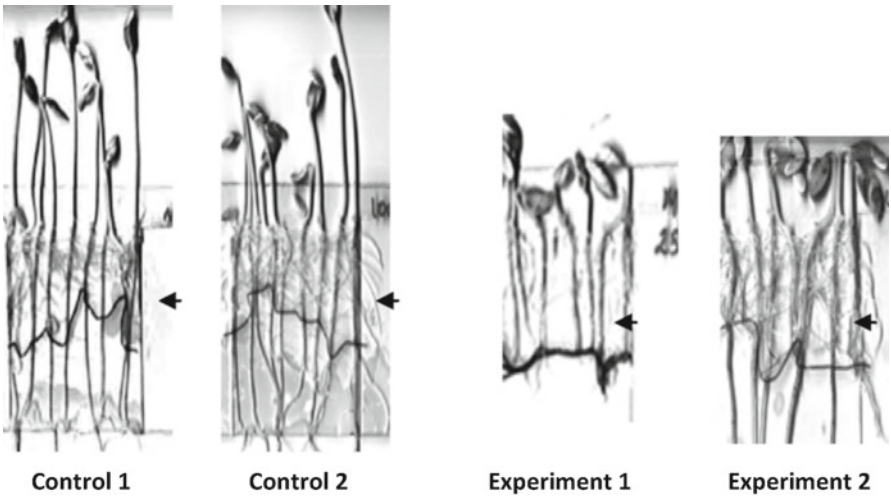

Fig. 10.4 Photos of 7-day old cucumber plantlets obtained from the seeds untreated (Control 1 and Experiment 1) and inoculated with a solution of preparative Reglalg (Control 2 and Experiment 2). Three days seedlings of Experiment 1 and Experiment 2 were treated with heat shock (5 min emersion in water with temperature 45 °C). The length of the roots at the time of heat shock is indicated by the *dark lines*, as emphasized with the *arrows*

unsaturated fatty acids, aldehydes, ketones, aldehyde-ketones and other bioactive components, prepared from algae in special conditions. Successful combination of such components assures a wide spectrum of applications. Plantlets of *Cucumis sativus* obtained from the seeds sprinkled with the preparation Reglalg (Control 2) had a small tendency to produce more developed roots system and less developed shoots in comparison with those obtained from seeds not treated with Reglalg. Under the influence of heat shock the growth of the roots of the plantlets obtained from seeds untreated with Reglalg was stopped completely (Experiment 1) and those obtained from seeds sprinkled with Reglalg continue to grow at the level comparable with that of control plants (Experiment 2). The preparation Reglalg could be considered to be an adaptogen. Under its actions the resistance of plants to heat shock increased (the area 2 of value of parameters of homeostasis maintaining became larger – Fig. 10.2). In supplemental experiments, it was shown that the beneficial effects of preparation Reglalg was partially dependent upon its protective action increasing resistance and as well due to cellular and molecular events immediately after heat shock (Reparation and Repopulation, Fig. 10.3). Thus the system theory helps biological researchers in analyzing a complex experimental results or designing new experiments.

From previous discussion, it is apparent that utilization of bioactive compounds by biosystems could be regarded as external signals (Fig. 10.1). The effect of each external signal is determined by the "target" of its action. If it influences the efficacy of MS, the product could be regarded mainly as a nutrient. If it activity is limited to RS, this products is a medication or adaptogen. Most obviously the bioactive compounds influence both the MS and RS and act concomitantly as a nutrition and

medication. It is important to state that the action of bioactive compounds also depend from the biosystem at which it influences. If this biosystem consists of a single organism, the influence could be as nutrition or/and medication. In the case of population or community in the biosphere, the impacts are primarily at the ecosystem level. Chemicals with antibiotic, insecticide, or even chemoattractant properties provide excellent examples of byproducts produced to improve the status of individual members of a community that may also directly or indirectly impact humans or be adapted to improve human health or food production. The widespread exploitation of biologically produced antibiotics discussed earlier in this presentation provides a prime example. A group of very interesting antibiotics that affects both plant microbe interactions as well as iron nutrition are siderphores. They are produced by bacteria and plants and provide an excellent example of substances affecting biosystem productivity through metabolic enhancement in that they are instrumental in controlling iron availability to both bacterial communities as well as higher plants [15, 16]. Additionally, these substances can be utilized to provide biocontrol of plant pathogens [17]. Another example of soil bacteria enhancing plant biomass production involves plant hormones. Soil microbes growing in the rhizosphere are a source of plant hormones [18]. The benefits of the hormones to the plant are clear, but the gains provided to the soil microbes are less obvious. The rhizosphere microbes benefit from this seemingly altruistic action by the fact that the plant is their primary nutrient and energy source. Thus, by stimulating plant biomass synthesis, the microbes gain an enhanced nutrient and energy resource.

Additional examples of bioproducts in maintaining extracellular homeostatic conditions would be production of natural pesticides by higher plants. A particularly interesting example of the latter is the production of pesticides by grass containing fungal endosymbionts [19]. These are a few examples of the pool of potentially useful bioproducts synthesized by plants, microbes, and animals that may be exploited to improve crop productivity and human health. These few examples provide evidence of the diversity of biological substances produced that control community structure, productivity and external threats. New interactions are continually being discovered, including some of the more recent studies of quorum sensing substances in biofilms, etc. From these limited examples it is clear that much more remains to be learned about the diversity and utility of bioactive compounds. Approaches discussed in this presentation are useful to classify and determine the potential role of mentioned substances as food or adaptogens for interacting biosystems of different level.

10.4 Conclusion

1. The biological functions of bioactive compounds as food, medicine and determining ecological equilibrium overlap considerably.
2. Implementation of the conception of biosystem and systemic approach is very useful in: (a) determining the “targets” of the influence of bioactive compounds on different levels of biosystems; (b)understanding the specificity of influences

of stress conditions on biosystems and changes of their purposes in dependence of stress level and duration; (c) elucidating the relative influence of bioactive compounds on biosystem as food, adaptogen and agents that determine ecological equilibrium; (d) elaborating effective systems of screening of bioactive compounds with adaptogen and ecological functions; (e) determining the biosystems resistance to different stress factors by appreciating the changes of redox potential under exposition to stress at different doses.

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Chapter 11
Ecological Potential of Plants

Edisher Kvesitadze, Tinatin Sadunishvili, and Georgi Kvesitadze

Abstract Elimination of contaminants from the environment by microorganisms of different taxonomic groups is a well established, genetically determined property, which has already been widely discussed. Until recently, plants, still occupying above 40 % of the world land, were considered as organisms just accumulating contaminants but having no potential to transform them into harmful compounds. Natural contaminations such as the emission of poisonous gases during a volcanic eruption and earthquakes, swamp poisoned evaporations, synthesis of toxic compounds by lower (microorganisms) and higher plants, etc., in comparison with the human contribution in the environmental contamination is much less impressive. As a result of urbanization, the unpredictable growth of industry and transport, production of chemicals for agriculture, military activities (explosives), etc., the concentration of anthropogenic toxicants in nature exceeds all the permissible standards. Analysis of experimental data of last two decades revealed the high ecological potential of plants. It has been exposed deep degradation processes proceeding in higher plants, in the majority of cases leading to the mineralization of contaminants. As a result, the enzymes carrying out oxidation and conjugation processes have been revealed and characterised; formation of anthropogenic contaminants conjugates with endogenous compounds and enzymes participating in this process has been shown. Although, still there are in plants some unlearned steps closely related to the contaminants multistage degradation process, authors are making an attempts for the evaluation of different aspects of plants ecological potential from the modern understanding, revealing the criterion for the evaluation of deviations under the action of contaminants in ultra structural architectonics of plant cells.

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11.1 Environmental Contamination and Role of Plants in Detoxification of Foreign Compounds

Natural contaminations, such as the emission of poisonous gases during a volcanic eruption and earthquakes, swamps poisoned evaporations, synthesis of toxic compounds by lower (microorganisms) and higher plants, etc., in comparison with the human contribution in the environmental contamination is much less impressive. As a result of urbanization, the unpredictable growth of industry and transport, production of chemicals for agriculture, military activities (explosives), etc. the concentration of anthropogenic toxicants spread in nature, especially in some regions, exceeds all the permissible standards. In spite of difficulties in quantitative, as well as in qualitative estimation, and having a tendency to be increased, the amount of spread out contaminants significantly exceeds annually one billion of tons. Most dangerous among these contaminants are considered as emergent because of their persistence, bioaccumulation, and toxicity along with our awareness of their prominent occurrence in the environment. In different ways, huge amounts of these hazardous substances or toxic intermediates of their incomplete transformations are accumulated in the different niches of biosphere, significantly affecting ecological balance. Lately, many ecological technologies have been elaborated, targeted to minimize the flow of toxic compounds to the biosphere and to control their level or state [11, 17]. Despite the some positive effect from the realization of these technologies (physical, chemical, mechanical etc.), the intensive flow of toxic compounds to the biosphere is still increasing [4, 10].

The international character of this problem being determined by global migration of contaminants (migration between soil, air and water, geographical, biotic, etc.) leads to distribution of toxic compounds of different structure and overall increasing the level of toxicity. Nevertheless, the plants kingdom members assimilate toxic compounds, removing them from the environment, naturally providing long-term protection and monitoring against their environmental dispersal. Obviously, microorganisms and plants represent the main power of nature permanently straggling for the maintaining of ecological balance.

11.1.1 Plants as Detoxifiers of Organic Toxicants

Plants being recently recognized as important ecological tool and in order to properly evaluate their detoxification potential should be emphasized according to following features: (i) higher plants simultaneously contact three main ecological niches: soil, water and air; (ii) well-developed root system of higher plants determines soil-plant-microbial interaction, representing unique process, significantly affecting the overall plant metabolism; (iii) large accumulating surface area of plant leaves (adaxial and abaxial), significantly exceeding in size the corresponding above ground surface located under the plant, permits the absorption of contaminants in a

big quantity from air via the cuticle and stomata; (iv) unique internal transportation system in both directions, distributing penetrated compounds throughout the entire plant; (v) autonomous synthesis of vitally important organics and extra energy during prolonged remediation process; (vi) existence of enzymes catalysing oxidation, reduction, hydrolysis, conjugation and other reactions of multistage detoxification process; (vii) large intracellular space to deposit heavy metals and conjugates of organic contaminants; (viii) functionalization and further transformation of organic contaminants in plant cells (conjugation, deep oxidation, etc.).

Xenobiotics (contaminants) to penetrate into a leaf should pass through the stomata, or traverse the epidermis, which is covered by film-like wax cuticle. Generally, stomata are located on the lower (abaxial) side of a leaf, and the cuticular layer is thicker on the upper (adaxial) side. Through stoma into leaves penetrate gases and liquids. The permeability for gases depends on the degree of opening of stomata apertures (4–10 nm), and for liquids the permeability depends on moistening of the leaf surface, surface tension of liquid and morphology of stomata. The majority of toxic compounds penetrate into a leaf as solutions (pesticides, liquid aerosols, etc.). The contaminants penetration into the roots essentially differs from the leaves. Substances pass into roots only through cuticle-free unsuberized cell walls. Therefore, roots absorb substances much less selectively than leaves. Roots absorb environmental contaminants in two phases: in the first fast phase, substances diffuse from the surrounding medium into the root; in the second they gradually distribute and accumulate in the tissues. The intensity of the contaminants absorption process, characterized by various regulations, depends on contaminants solubility, molecular mass, concentration, polarity, pH, temperature, soil humidity, etc. [9, 11].

Nowadays there are experimental data obviously demonstrating that plants activate a definite set of biochemical and physiological processes to resist the toxic action of contaminants by the following mechanisms: (i) excretion; (ii) conjugation of contaminants with intracellular compounds following by compartmentalization of conjugates in cellular structures; and (iii) decomposition of environmental contaminants (or their significant part) to standard cell metabolites or their mineralization.

Commonly, plants gradually degrade entering cells organic contaminants to avoid their toxic action. According to contaminants assimilating potential plants sometimes are differing up to four orders of magnitude that allowed to classifying plants as strong, average and weak absorbers of different structure contaminants. For instance the most active assimilators uptake up to 10 mg of benzene per 1 kg of fresh biomass per day, the assimilation potential of the weak absorbers is measured in hundredths of milligram [19]. The fate of entered plant cell contaminants depends on their chemical nature, external temperature, variety of plants and phase of vegetation, etc. The simplest pathway of entered the plant cell organic contaminants is excretion. The essence of excretion is that the toxicant molecule does not undergo chemical transformation, and being translocated through the apoplast, is excreted from the plant. Xenobiotics (contaminants) elimination is rather rare and takes place at high concentrations of highly mobile (phloem-mobile or ambi-mobile) xenobiotics.

In the majority of the cases, contaminants being absorbed and penetrated into plant cell undergo enzymatic transformations leading to the increase of their

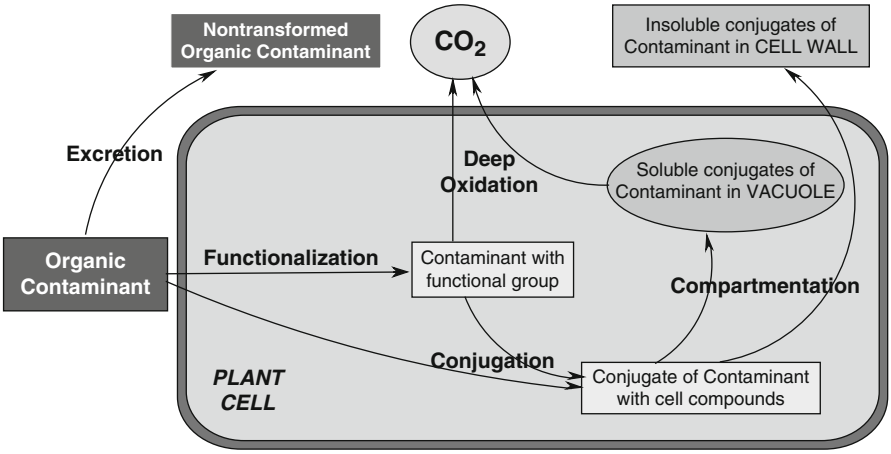

Fig. 11.1 The main pathways of organic contaminant transformation in plant cells

hydrophilisity-process simultaneously accompanied by decreasing of toxicity. Below are presented successive phases of contaminants initial transformations in accordance to Sanderman's green liver concept [16] (Fig. 11.1).

Functionalization is a process whereby a molecule of a hydrophobic organic xenobiotic acquires hydrophilic functional group (hydroxyl, carboxyl, amino, etc.) as a result of enzymatic oxidation, reduction, hydrolysis, etc. Due to the introduction of functional group the polarity and correspondingly reactivity of the toxicant molecule is enhanced. That promotes an increase of intermediates affinity to enzymes, catalyzing further transformation. *Conjugation* takes place as a basic process of phytoremediation and is determined by formation of chemically coupled contaminant to endogenous cell compounds (proteins, peptides, amino acids, organic acids, mono-, oligo-, polysaccharides, lignin, etc.) forming of peptide, ether, ester, thioether or other type covalent bonds. Intermediates of contaminants initial transformations or contaminants themselves possessing functional groups capable of reacting with intracellular endogenous compounds are susceptible to conjugation.

Commonly, the main part of the toxicant molecules undergoes conjugation and only a small amount is deeply degraded (0.1–2 % depending on contaminants structure). Conjugation is a wide spread defence mechanism in higher plants especially in cases when penetrated into plant cell concentration of the contaminants exceeds the plant's transformation (decomposition) potential. Increased amount of deep degradation to regular plant sell metabolites, or CO_2 and water is achieved in case of linear, low molecular structures of contaminants [11, 18]. The toxicity of conjugates compared to parent compounds is decreased due to binding with non-toxic cellular compounds. Conjugates are kept in a cell for a certain period of time without causing visible pathological deviations in cell homeostasis. Conjugates formation also gives the plant cell extra time for the internal mobilization, induction of enzymes responsible for contaminants further transformation. Relatively quickly,

after the termination of plant incubation with the contaminant, conjugates are no longer found in plant cells.

Some attempts have been made by authors (unpublished data) to estimate different plant (soybean, ryegrass) cells potential to accumulate conjugated benzene in their cells in case of toxicant saturation. In spite of incomplete information it was suggested that for genetically non-modified plants it could be, as a minimum, several molecules of contaminant conjugates per each plant sell. Although conjugation is one of the most widely distributed pathways of plant self-defence, it cannot be assumed as energetically and physiologically advantageous for the plant metabolism process. Firstly formation of conjugates leads to the depletion of vitally important cellular compounds, and secondly unlike deep degradation, formation of conjugates is maintaining contaminants basic molecular structure, and hence results only in partial and provisional decreasing of its toxicity.

Compartmentalization is, in most cases, the final step of conjugates processing temporary (short or long) storage of conjugates in defined compartments of the plant cell takes place. Soluble conjugates of toxic compounds (coupled with peptides, sugars, amino acids, etc.) are accumulated in cell structures (primarily in vacuoles), while insoluble conjugates (coupled with, lignin, starch, pectin, cellulose, xylan) are moved out of the cell via exocytose in the apoplast being accumulated in cell wall [16]. The compartmentalization process is analogous to mammalian excretion, essentially removing toxic part from metabolic tissues. The major difference between detoxification in mammals and plants is that plants do not have a special excretion system for the removal of contaminants conjugates from the organism. Hence they use a mechanism of active transport for the removal of the toxic residues away from the vitally important sites of the cell (nuclei, mitochondria, plastids, etc.). This active transport is facilitated and controlled by the ATP-dependent glutathione pump [12] and is known as "storage excretion" [2].

The described above pathway of toxic compound processing i.e., functionalization → conjugation → compartmentalization, is well illustrated by the processing of anthropogenic contaminants of different structures. One of such examples demonstrating the transformation of organochlorine pesticides is the hydroxylation of 2,4-D (Fig. 11.2) followed by conjugation with glucose and malonyl residues and deposition in vacuoles [15].

11.1.2 Enzymes

Anthropogenic organic toxicants decomposition processes are closely related to many aspects of higher plants cellular metabolism. In prolonged and multifunctional detoxification processes quite a few enzymes are actively involved. According to catalyzed reactions they are directly or indirectly participating in detoxification process.

Transformations of contaminants during functionalization, conjugation and compartmentation are of enzymatic nature. It is remarkable that due to their unusual flexibility in the absence of xenobiotics, in plant cell these enzymes catalyze

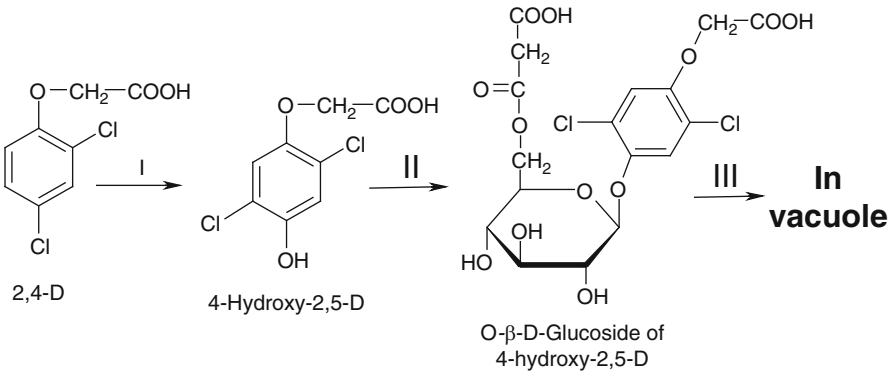

Fig. 11.2 2,4-D transformation for deposition in vacuoles

reactions typical for regular plant cell metabolism. Based on multiple literature data the following enzymes directly participate in the transformation process of anthropogenic contaminants: (i) oxidases, catalyzing hydroxylation, dehydrogenation, demethylation and other oxidative reactions (cytochrome P450-containing monooxygenases, peroxidases, phenoloxidases, ascorbatoxidase, catalase, etc.); (ii) reductases, catalyzing the reduction of nitro groups (nitroreductase); (iii) dehalogenases, splitting atoms of halogens from halogenated and polyhalogenated xenobiotics; (iv) esterases, hydrolyzing ester bonds in pesticides and other organic contaminants.

Conjugation reactions of contaminants in plant cell are catalyzed by transferases: glutathione S-transferase (GST), glucuronozyl-O-transferase, malonyl-O-transferase, glucosyl-O-transferase, etc. Compartmentalization of intermediates of contaminants transformation-conjugates takes place under the action of ATP-binding cassette (ABC) transporters [3]. Depending on the structure of the contaminant some other enzymes may also be involved in their degradation process.

Prolonged in time cellular decomposition of contaminants involves participation of enzymes providing plant cell with extra energy needed for the defence processes, induction of the enzymes, and provision of cells by vitally important secondary metabolites. Enzymes involved in these and similar processes obviously indirectly participate in the detoxification of contaminants.

The correlation between the penetration of organic contaminants (alkenes, aromatic hydrocarbons, polycyclic aromatic hydrocarbons) in plant cells and the corresponding changes in the activities of enzymes participating in energy supply (malate dehydrogenase) and nitrogen metabolism (glutamate dehydrogenase, glutamine synthetase) has been revealed. As it has been shown the activities of the enzymes are highly affected by xenobiotics concentration, exposure time and mode of illumination [9].

Ecologically the most advantageous pathway of organic contaminants transformation in plants is their deep oxidative degradation. In higher plants mainly the following enzymes are responsible for this process: cytochrome P450-containing

monooxygenese, peroxidase and phenoloxidase. To correctly evaluate the universality of the action of these enzymes, responsible for the degradation of different structure organic contaminants, some of their specificities should be emphasized (Table 11.1).

11.1.3 Ultrastructure

To evaluate the ecological potential of plants, the data proving the responses at the level of cell ultrastructure under the action of contaminants, as the most precise indications of plants exploitation, should also be emphasized. Undoubtedly, penetration even a small concentrations of contaminants into plant cells leads to invisible, but most often measurable deviations in cell metabolic processes such as: induction of enzymes, inhibition of some intracellular metabolic processes, change the level of secondary metabolites, etc. The existence in plant cell contaminants in increased concentrations provokes clearly noticeable deviations in cells ultrastructural organization. It has been shown that the complex of changes and alterations in the main metabolic processes of plant cell elicited by organic pollutants (pesticides, hydrocarbons, phenols, aromatic amines, etc.) are connected with the deviations of cell ultrastructural architecture. The sequence and deepness of the destruction in plant cell organelles are determined by the variety of plant, chemical nature, concentration and duration of the contaminant action, etc. [1, 20]. This course of events has been experimentally demonstrated by authors in a number of various higher plants exposed to different ^{14}C-labelled toxic compounds. In these experiments due to the penetration, movement and localization of contaminants in plant cells changes in ultrastructural organization has been shown. Apparently, the negative affects of toxic compounds on cell ultra structure, depending on its concentration, could be divided on two types, being different for each contaminant and plant: (i) metabolic, which is digested by the plant in spite of insignificant negative effect by the mobilization of plants internal potential; (ii) lethal, leading to indigestible deviations and to the plant death.

Figure 11.3 shows maize root apex cells exposed to ^{14}C-nitrobenzene action, its penetration across the plasmalemma and localization in subcellular organelles. Studies of penetration of ^{14}C-labelled xenobiotics into the plant cell indicate that labelled compounds at the early stages of exposure (5–10 min) are detected in the cell membrane, in the nuclei and nucleolus (in small amounts), and, seldom, in the cytoplasm and mitochondria. As a result of prolonged exposure the amount of a label significantly increases in the nucleus, at the membranes of organelles, in tonoplasts, and further in vacuoles [21], i.e. xenobiotic becomes distributed in most of subcellular organelles, but ultimately there is a tendency of contaminants primary accumulation in vacuoles.

Obviously plants, as remediators, for a long time the most effectively act at low and shallow contamination of soil and air, when no significant changes in cell ultrastructure take place. Planting of almost any kind of vegetation, including

Table 11.1 Plants oxidative metalloenzymes

Enzyme	Physiological function	Existence in cell	Localization	Specificity to toxicants	Limiting factors	Stability
Cytochrome P450 containing monooxygenase	Participation in a number of intracellular synthesizing reactions	Small amount, inductive nature	Endoplasmatic reticulum, cytosole	Very high affinity to nonpolar toxicants	NADPH NADH	Labile, being inactivated during substrate oxidation
Peroxidase	Hormonal regulation, lignification, response on stress, removing of peroxides	Large amount, inductive nature	Cell wall, vacuoles, cytosole, tonoplasts, plastids, plasmalemma	Affinity to aliphatic compounds	H_2O_2 or organic hydro-peroxides	Stable
Phenoloxidase	Oxidative transformation of phenols, lignification, cell defence reactions	Large amount, presents in latent form too, inductive nature	Chloroplasts, cell wall, cytosole, tonoplasts	Affinity to aromatic compounds	Endo-genous phenols	Stable

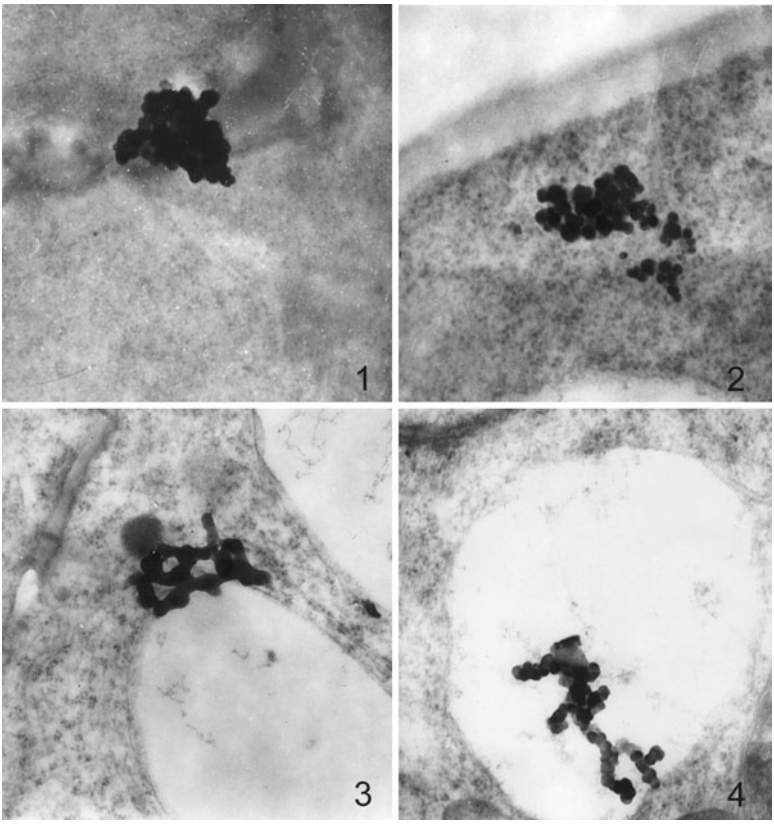

Fig. 11.3 Electron micrographs showing the penetration and movement of ^{14}C-labelled nitrobenzene (0.15 mM) in a maize root apex cell [21]. The xenobiotic penetrated through the plasmalemma (*1*), moved to the cytoplasm (*2*) and thereafter translocated into vacuoles (*3*, *4*). Magnification: *1* – 48,000×; *2* – 36,000×; *3* – 50,000×; *4* – 30,000×

agricultural vegetation is beneficial for environment. However, in order to make the exploitation of most ecological potential of each particular plant, the selection should be carried out according to plants potential to assimilate/accumulate toxic compounds of different structure.

Phytoremediation is a unique cleanup strategy. The realization of phytoremediation technologies implies the planting on a contaminated area with one or more specific, previously selected plant species with the potential to extract contaminants from soil. A precise survey of the vegetation *in situ* should be undertaken to determine what species of plants would have the best growth at the contaminated site. Based on the number of experimental results including the use of labeled xenobiotics and electron microscopic observations, the deep degradation of anthropogenic contaminants in higher plants could be considered as narrow but permanently working pathway having much less potential than conjugates formation process, especially, in case of contaminants saturation.

Transgenic plants have also been studied in connection with degradation of several (some) particular contaminants. For this purpose the widely distributed explosive TNT has generally been chosen. In order to increase the degradability of TNT and similar compounds, the transgenic plants (several) contained the gene of bacterial enzyme (pentaeritrole tetranitrate reductase, EC 1.6.99.7) were received [6]. Transgenic tobacco has been analysed for its ability to assimilate the residues of TNT and trinitroglycerine. Seedlings of the transgenic plants extracted explosives from the liquid area much faster, accomplishing denitration of nitro groups, than the seedlings of common forms of the same plants, in which growth was inhibited by the contaminants [7]. Transgenic tobacco thus differed substantially from the common plant by its tolerance, fast uptake and assimilation of significant amounts of TNT. Analogous experimental results have been obtained with other plant species [8].

There are dozens of publications concerning successful improvement of plant detoxification abilities by cloning the genes of transferases and oxidases, which intensively participate in contaminants transformation processes [13, 14].

Obviously, attempts to improve artificially ecological potential of higher plants will be continued, and the results will be the more substantial from the viewpoint of their eventual practical realization. The positive effect of these investigations could be much more impressive if all aspects of the quite complicated and multistage detoxification process would be better elucidated with regard to plants physiology and biochemistry. Such information would allow the creation of more rational and effective strategy for the gene engineering potential application.

Finally in correlation with plants degradation potential (greatly depending on the plant variety) is determined by eliminating toxicants through metabolic degradation or by exocitos mechanism. Owing to the still wide terrestrial and aquatic distribution of plants we should consider these organisms as a very important biological instrument having tremendous ecological potential.

11.2 Conclusion

- Organic toxicants undergo deep oxidation in plants leading to mineralization.
- Enzymes conducting oxidation and conjugation of absorbed contaminants have been revealed and characterized.
- Enzymatic conjugation of contaminants with endogenous compounds has been demonstrated.
- Penetration and movement of ^{14}C-nitrobenzene in plants has been established.

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Chapter 12
The Protective Effects of Natural Polyphenolic Complexes of Grape Wine on Organisms Exposed to Oxidative and Nitrosative Stress Under Diabetes Mellitus

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Abstract Peripheral nerve damage is a significant complication of diabetes mellitus. The ingestion of polyphenols available naturally in a variety of plant products may provide impressive protection against such damage. The natural polyphenol complex of grape wine has a significant anti-diabetic effect. It can protect against dehydration at the level of the whole organism and deter free radical-induced damage to the sciatic nerve, the spinal cord, kidney and retina. The levels of nitrosylated and PARylated proteins can be restored to near control levels by the extract. The biochemical mechanisms of action of the natural polyphenol complex of grape wine require further research, but may be considered as a valuable therapeutic approach for the treatment of diabetic complications.

12.1 Introduction

Diabetes Melitus (DM) has reached epidemic proportions with 6–8 % of the population of the developed countries suffering from the disease. The statistical research conducted by the World Health Organization (WHO) predicts an increase in the number of diabetics in 2025 to up to 380 million people. In Ukraine, more than one million patients with DM are registered. The number of children with diabetes under the age of 5 from 1985 till 2009 has increased by seven times. Today, in Ukraine, more than 8,000 children are affected by DM. The growth in the incidence of diabetes, the

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early disability of the working population causing a significant economic damage to the country, reduced quality of life and a shortening of its duration in patients with diabetes have attracted the attention of scientists to this problem.

Hyperglycemia is the main established pathogenetic factor for the development of DM, which occurs against the background of insulin deficiency, and is the prime determinant of the diagnosis of type 1 DM. Under hyperglycemia, the violation of the electron transport chain leads to the overproduction of superoxide anion, which, interacting with other reactive oxygen species (ROS), activates free radical oxidation leading to oxidative stress (OS) [1]. Oxidative stress, in its turn, causes the disruption of cellular homeostasis, accumulation of molecules with altered structure, damage to the structure of lipids, proteins and DNA. In response to DNA damage, the nuclear enzyme poly(ADP-ribose) polymerase (PARP-1) is activated. Poly(ADP-ribosyl)ation of numerous nuclear proteins takes place causing significant energy depletion of cells. Under certain conditions, it can cause cell death [22]. It has been proven that PARP-1 can poly(ADP-ribosyl)ate glyceraldehyde 3-phosphate dehydrogenase (the enzyme of glycolysis) that leads to the inhibition of glycolytic glucose utilization at the level of glyceraldehyde 3-phosphate formation with subsequent accumulation of the intermediate products of glycolysis. This activates a number of signaling and metabolic pathways, including polyol and hexosamine pathways, activation of PKC and accumulation of methylglyoxal, uncharacteristic for normal physiological conditions. Such changes in metabolism (e.g. accumulation of fructose, sorbitol, methylglyoxal, advanced glycation end products, etc.) is a trigger for the development of diabetic angiopathy, such as damage of retinae (retinopatia), kidneys (nephropatia), the peripheral nervous system (neuropatia), etc.

Activation of alternative pathways of glucose utilization leads to an increase in nonenzymic glycosylation under diabetic conditions. Under the interaction of AGEs with specific receptors (RAGES), the cascade of mechanisms is activated, causing increased gene expression that encodes a number of proinflammatory cytokines (TNF-α, IL 1,6), vasoconstrictors – endothelin 1, adhesion molecules (ICAM-1, VCAM-1) and growth factors. They disturb the function of blood vessels and contribute to premature development of atherosclerosis and inflammatory processes [19], induce the super-activity of the local renal renin-angiotensin system, help to increase glomerular blood pressure and filtration rate, etc. The latter injuries cause the development of microalbuminuria and microproteinuria. Accordingly, we can assume that the effect of OS and AGEs primarily leads to the development of diabetic nephropathy (DN).

Diabetic nephropathy occurs as a result of a complex variety of pathological processes which are formed primarily in the capillaries and small vessels of the kidneys. Like all microangiopathies, this disease is realized through the emergence and progression of endothelial dysfunction.

As well known, kidney glomerulus consists of endotheliocytes that cover the capillaries from the inside and podocytes that cover the capillaries from the outside, providing a filter barrier and mesangial cells. These structures are the elements of smooth muscle tissue that are located around the capillaries and involved in the regulation of blood flow velocity. Experimental DM (EDM) is characterized by podocyte apoptosis

that is accompanied by a sharp decrease in their number. All of these changes can lead to a thickening of the filtering barrier with the involvement of proteins, (collagen IV, in particular) with further progression of fibrogenesis until the complete loss of the physiological function of the glomerulus. This is the reason why inflammatory processes in the glomerulus are accompanied by an increase in body size and weight [15].

DM retinopatia at early stages is characterized by a partial increase in permeability of retinal vessels, the loss of vessel pericytes followed by a thickening of the vessel walls, their degeneration, poor blood circulation in the eye and the development of hypoxia [8]. A decrease in perfusion of capillaries and the resultant hypoxia leads to neovascularization, excessive development of abnormal endothelium and the accumulation of collagen. Angiogenesis is accompanied by the modification of endothelium and basement membrane degradation. All of this eventually leads to retinal detachment [4].

Accumulation of fructose, sorbitol, methylglyoxal, and the advanced glication end products is a trigger for the development of DN. Initial metabolic changes occurring in the nerve fibers cause disruption of their functions, and eventually lead to changes in their structure. In particular, the activation of the polyol pathway in nervous tissue with the characteristic accumulation of sorbitol and fructose, which consistently causes a decrease in activity of Na^+, K^+-ATPase and the level of mioinozytol, leads to the retention of Na^+ and water, swelling of the myelin sheath, its further demyelination and reduction of motor and the sensory nerve conduction velocity of the peripheral nervous system.

Simultaneously, in parallel with it, there is a total destruction of the organism by ROS that, in addition to DNA damage, causes damage of axon membrane structures of peripheral nerve fibers and, as a result, damage of the nerve cell structures and functions. In addition to direct damaging effects, the accumulation of ROS affects the energy metabolism in neurocites and development of endoneurial hypoxia. Such a comprehensive total damage causes demyelination and degeneration of the nerve fibers and reduces their functional activity. Under these conditions, the antioxidant system of the organism plays an important role, particularly its enzymatic component, the main task of which is the neutralization of free radicals. Under DM it has reduced activity. Strengthening of the antioxidant system by exogenous antioxidants could provide a protective effect to all body systems.

Research into natural antioxidants – polyphenol complexes of grape wine, including proanthocyanidins, derived flavan-3-ols and several other derivatives of flavonoids, which are effective in preventing cardiovascular diseases, have become promising recently [16]. It is known that polyphenols of grape wine are able to interact with plasma proteins and cellular elements of blood, prevent premature oxidation of their molecular complexes, which occurs under oxidative and nitrosative stress. Significant bactericidal and antiviral effect of these substances has been shown [5]. The protective effects of the polyphenols complexes of grape wine on some systems and organs under oxidative stress and during the metabolic syndrome have been detected [17, 18].

The protective properties of natural grape polyphenol complexes under streptozotocin-induced DM and their effect on the enzymic antioxidant system during the development of angiopathies have been sparsely investigated. The goal of our work,

therefore, has been to investigate the protective antioxidant effect of polyphenol complexes of grape wine on the enzymatic antioxidant system, its ability to prevent the formation of nitrotyrosine modified proteins and activation of PARP-1 in different tissues of rats with streptozotocin-induced DM.

12.2 Materials and Methods

All animal care and procedures were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes Directive of 24 November 1986 (86/609/ECC) and were approved by Bioethics Committee of the Ivan Franko National University of Lviv Protocol for Animal Studies, Lviv, Ukraine. Male Wistar rats, of 190–210 g body weight, were fed a standard rat chow and had access to water *ad libitum*.

The specimen of natural polyphenols complexes of grape wine was received by evaporation of red wine in rotary evaporators LABOROTA 4000 (Heidolph, Germany). The red wine was made by the classical technology from Cabernet Sauvignon (clone C337/S04C3) grapes and contained phenolic compounds (2,309 mg/L), proanthocyanidines (936 mg/L) and pigment polymers (444 mg/L).

The rats were separated into four groups of seven animals each: Group 1 – normal untreated control, Group 2 – polyphenol treated, Group 3 – streptozotocin (STZ) treated, and Group 4 – polyphenol and STZ treated. The STZ treatment was a single i.p. injection of 50 mg STZ/kg body weight. The specimen treatment was an oral dose (300 ml/70 kg body weight/day) that constituted 23.5 mg/kg body weight/day administered daily for 2 weeks prior to the STZ injection and daily for 4 weeks after the STZ injection. Group 2 received a specimen of natural polyphenol complexes of grape wine for 6 weeks. Blood samples for glucose measurements were taken from the tail vein 72 h after the STZ injection and the day prior to the study termination. All of the rats with blood glucose of 14 mmol/L or more were considered diabetic.

The animals were sedated by CO_2 and immediately sacrificed by cervical dislocation. One sciatic nerve, an eye and a part of the spinal cord and dorsal root ganglia (lumbar and sacral nerve roots) from each rat were fixed in 10 % neutral-buffered formalin for further assessment of poly(ADP-ribose) by conventional immunohistochemistry. The second sciatic nerve, eye bowl and another part of the spinal cord and dorsal root ganglia from each rat were immediately frozen in liquid nitrogen for subsequent Western blot analyses of the protein 3-nitrotyrosine content.

To assess nitrosylated proteins by Western blot analysis, tissue samples were transferred to an extraction buffer (1:10 wt/vol), containing 50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 0.1 % sodium dodecyl sulfate (SDS), 1 % NP-40, 5 mM EDTA, 1 mM EGTA, 1 % sodium deoxycholate, and the protease/phosphatase inhibitors: leupeptin (10 μg/ml), aprotinin (20 μg/ml), benzamidine (10 mM), phenylmethylsulfonyl fluoride (1 mM), sodium orthovanadate (1 mM), and homogenized on ice. The homogenate was sonicated (3×5 s) and centrifuged at 14,000 *g* for 20 min. All the

afore-mentioned steps were performed at 4 °C. The lysates (20 μg of total protein per lane) were mixed with the equal volume of sample-loading buffer, containing 62.5 mM Tris–HCl (pH 6.8), 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol and 0.025 % bromophenol blue, heated at 95 °C for 5 min, separated on 10 % SDS-PAGE and transferred onto a nitrocellulose membrane.

Free binding sites were blocked in 2 % (w/v) bovine serum albumin (BSA) in phosphate buffer saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.3, and 0.05 % Tween 20, for 1 h, after which nitrotyrosine antibodies were applied for 2 h. The horseradish peroxidase-conjugated secondary antibody was then applied for 1 h. After extensive washing, protein bands detected by the antibodies were visualized with the ECL Detection Reagents (Amercham, USA). The total content of all nitrosylated proteins was quantified by densitometry (Gel Pro Analyzer 3.1, Media Cybernetics USA). Membranes were then stripped in the 25 mM glycine-HCl, pH 2.5 buffer containing 1 % SDS, and reprobed with β-actin antibody to confirm equal protein loading.

All sections were processed by a single investigator and evaluated blindly. Poly(ADP-ribose) immunoreactivities in the sciatic nerve, spinal cord, dorsal root ganglia neurons (DRG) and retina were assessed by immunohistochemical techniques (light and fluorescent microscopy). Tissues were fixed in 10 % neutral-buffered formalin and 7 μm sections were prepared from paraffin embedded tissues. Paraffin sections were deparaffinized in xylene, hydrated in decreasing concentrations of ethanol and washed in water. Optimal staining was achieved with antigen retrieval solution.

For immunofluorescent histochemistry, non-specific binding was blocked with the 10 % of normal goat serum and 1 % BSA in PBS buffer in the humidity chamber for 1 h. Firstly, mouse monoclonal antipoly(ADP-ribose) antibody was diluted 1:100 in 1 % BSA in PBS, and applied overnight at 4 °C in the humidity chamber. Secondly, Alexa Fluor 488 goat anti-mouse antibody was diluted 1:200 in PBS and applied for 2 h at room temperature. Sections were mounted in Prolong Gold Antifade Reagent.

For light microscopy, endogenous peroxidase was quenched with 0.3 % H_2O_2 for 20 min. Non-specific binding was blocked with the 10 % of normal goat serum and 1 % BSA in PBS buffer in the humidity chamber for 1 h. Then, avidin/biotin blocking kit (Vector Laboratory Inc., USA) was used to block endogenous biotin and avidin. Mouse monoclonal anti-poly(ADP-ribose) antibody (diluted 1:100 in 1 % BSA in PBS) was applied overnight at 4 °C in the humidity chamber. The detection was performed using the secondary biotin-conjugated goat anti-mouse antibody (diluted 1:200 in PBS and applied for 2 h at room temperature) and Vectastain Elite ABC kit (Vector Laboratory Inc., USA). The positive signals were visualised with the 3,3′-diaminobenzidine (DAB substrate kit, Vector Laboratory Inc., USA). The sections were counterstained with hematoxylin, dehydrated and mounted in the micromount mounting medium (Surgipath Medical Ind., Inc, USA).

Negative controls included elimination of the primary antibody. At least ten fields of each section were examined to select one representative image. Representative images were microphotographed and the intensity signal was quantified with ImageJ 1.32 software (National Institutes of Health, USA). Low power observations of

Table 12.1 Body weight and blood glucose concentration in control and diabetic rats with or without polyphenol complexes consumption (mean ± s.e.m, n = 5–7)

Indicators / Rodent group	Blood glucose (mmol/l)		Body weight (g)	
	Initial§	Final	Initial§	Final
C	5.7 ± 0.5	6.2 ± 0.5	206.0 ± 16.1	279.0 ± 19.6
C + Pol	5.9 ± 0.2	6.3 ± 0.2	202.0 ± 17.3	276.0 ± 20.0
D	18.5 ± 0.9**	26.8 ± 2.2**	200.0 ± 6.8	209.0 ± 30.0**
D + Pol	20.0 ± 0.3**	26.9 ± 1.9**	198.0 ± 6.4	234.5 ± 10.5*,#

Notes: *C* control, *C* + *Pol* control + specimen of polyphenols, *D* diabetic, *D* + *Pol* diabetic + specimen of polyphenols. *P < 0.05 and **P < 0.01 vs. control, #P < 0.05 vs. control and diabetic rats without polyphenol specimen consumption. § – third day after induction of diabetes

sciatic nerve, spinal cord, DRG and retina sections stained for poly(ADP-ribose) were made using a Nikon Optiphot 2 imaging microscope. Color images were captured with a DCM310 microscope CMOS camera. Low power images were generated with a 40× acroplan objective using the automatic capturing feature of the ScopePhoto software.

Tissues homogenization was carried out using hand homogenizers in the presence of 0.1 M phosphate buffer (1:10 wt/vol) pH 7.0 on ice. Homogenized samples of spinal cord were centrifuged for 30 min at 14,000 g at 4 °C. After the removal of a thin lipid layer, it was recentrifuged for 15 min at 10,000 g at 4 °C. Samples of sciatic nerves and DRG were centrifuged for 20 min at 10,000 g at 4 °C.

The activity of superoxide dismutase (SOD) was determined by Chevari method, catalase by the Corolyk method, glutathione peroxidase by the Moin method, glutathione reductase by the Goldberg method. The MDA level was analyzed with 2-thiobarbituric acid by the Timyrbulatov method. The concentration of protein was determined by the Lowry method.

Differences among experimental groups were determined by ANOVA and the significance of between-group differences was assessed by Student–Newman–Keul's multiple range test. Significance was defined at $P \leq 0.05$.

12.3 Results and Discussion

At the end of the experiment, the final body weight of control rats and rats consuming the specimen of natural polyphenol complexes of grapes increased by 36 % compared with the body weight of animals before the experiment (Table 12.1). In contrast to the control groups, the body weight of rats with diabetes slightly decreased. At the same time, the body weight of rats with diabetes that consumed the specimen increased by 18 %. In groups of rats with STZ-induced diabetes, this phenomenon can be explained by the "overproduction" of urea. The latter was excreted from the body by the osmotic diuresis together with corresponding amount of water, K^+ and Na^+ ions [13]. This process leads to dehydration, which further increases due to the release of free fatty acids from adipocytes followed by their conversion to ketone

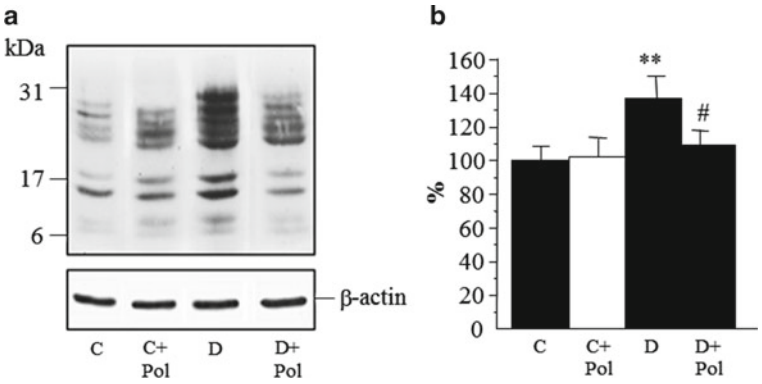

Fig. 12.1 Representative Western blot analyses of nitrotyrosine-modified proteins in the retinae (**a**) of control and diabetic rats with and without polyphenol consumption. Total nitrotyrosine content (**b**). Total nitrotyrosine protein content in control rats is taken as 100 %. Equal protein loading was confirmed with β-actin antibody. *C* control groups, *D* diabetic groups, *Pol* polyphenols complexes of grape wine. The data are expressed as mean ± s.e.m., n = 5–7. **p < 0.01 vs. controls; #p < 0.05 vs. diabetic rats without polyphenols consumption

bodies (acetoacetate and β-hydroxybutyrate) leading to ketoacidosis. The ketone bodies "provoke" an increase in osmotic diuresis and loss of electrolytes [14]. Such changes of the level of body water affect the general metabolism and body weight. The indices of body weight in groups of rats with STZ-induced diabetes that consumed the specimen increased significantly to control values.

The results presented in Table 12.1, comparing indices at the beginning and the end of the experiment, show an increase in hyperglycemia in the groups affected by DM and the absence of any glycemic effect of the polyphenolic complexes.

Other researchers have shown that relatively high doses of resveratrol, which are basically a part of the polyphenol complex of grape wine, significantly reduced glucose levels to normal in rats with STZ-induced diabetes [23]. The results indicate that a decrease in the level of blood glucose of diabetic animals depends upon the variety of grapes and the amount of the dose.

Reactive nitrogen compounds, including peroxynitrite play a crucial role in the pathogenesis of diabetes and its complications [9, 22]. It has been recently revealed that nitrotyrosine (NT), a marker of oxidative-nitrosative stress, is accumulated in vascular endothelium, myocardium [22], retina [7], blood flow [2], kidneys, and the peripheral nervous system of diabetic rodents as well as in obese fatty Zucker rats, Zucker diabetic fatty rats and leptin knockout (ob/ob) mice [3, 20]. These data indicate that peroxynitrite-induced injuries are present in the early and late stages of Type 1 and Type 2 DM and during the prediabetic stages. Peroxynitrite is a powerful oxidant in the pathogenesis of diabetic complications [10, 20], including endothelial [22], peripheral and autonomic neuropathy [10], and retinopathy [12].

Western blot analysis showed an increase in the content of NT in the retina of rats with DM by 38 % compared with control (Fig. 12.1). Nitrosative stress was partially normalized by the consumption of polyphenol complexes of grape wine, and the

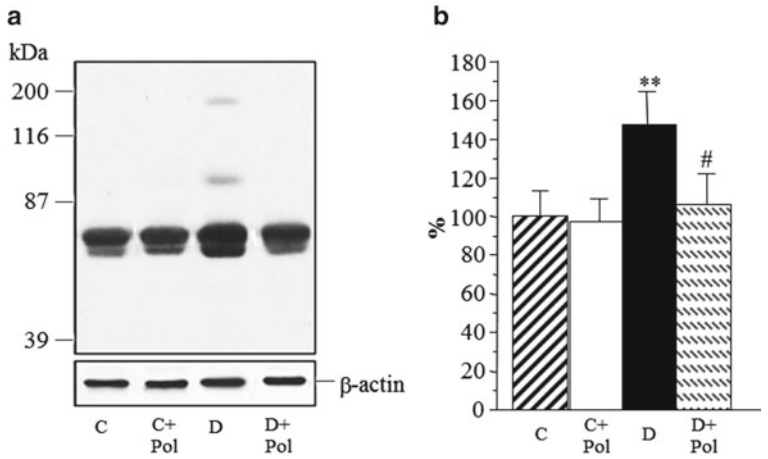

Fig. 12.2 Representative Western blot analyses of nitrotyrosine-modified proteins in the sciatic nerve (**a**) of control and diabetic rats with and without polyphenols consumption. Total nitrotyrosine context (**b**). Total nitrotyrosine protein content in control rats is taken as 100 %. Equal protein loading was confirmed with β-actin antibody. *C* control groups, *D* diabetic groups, *Pol* polyphenols complexes of grape wine. The data are expressed as mean ± s.e.m., n = 5–7. **p < 0.01 vs. controls; ##p < 0.01 vs. diabetic rats without polyphenols consumption

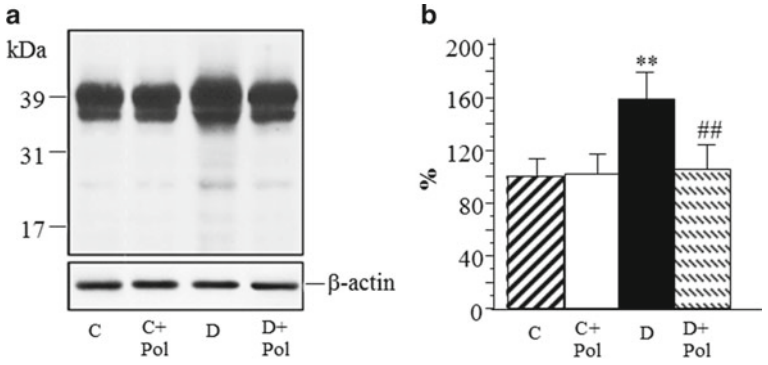

Fig. 12.3 Representative Western blot analyses of nitrotyrosine-modified proteins in the DRG (**a**) of control and diabetic rats with and without polyphenols consumption. Total nitrotyrosine context (**b**). Total nitrotyrosine protein content in control rats is taken as 100 %. Equal protein loading was confirmed with β-actin antibody. *C* control groups, *D* diabetic groups, *Pol* polyphenols complexes of grape wine. The data are expressed as mean ± s.e.m., n = 5–7. **p < 0.01 vs controls, ##p < 0.01 vs. diabetic rats without polyphenols consumption

recovery of the level of nitrotyrosine was almost to control levels (P < 0.05). The similar increase in the content of nitrotirosine-modified proteins was observed by 48 % in the sciatic nerve (Fig. 12.2), by 60 % in the DRG (Fig. 12.3) and by 40 % in the spinal cord (Fig. 12.4) of the rats with diabetes. After consumption of the specimen of natural polyphenol complexes by rats with DM, we observed a partial

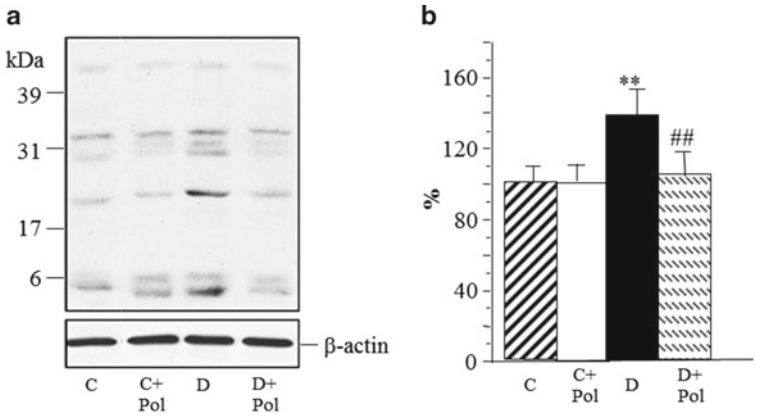

Fig. 12.4 Representative Western blot analyses of nitrotyrosine-modified proteins in the spinal cord (**a**) of control and diabetic rats with and without polyphenols consumption. Total nitrotyrosine context (**b**). Total nitrotyrosine protein content in control rats is taken as 100 %. Equal protein loading was confirmed with β-actin antibody. *C* control, *D* diabetic groups, *Pol* polyphenols complexes of grape wine. The data are expressed as mean ± s.e.m., n = 5–7. **$p < 0.01$ vs. controls, $^{\#\#}p < 0.01$ vs. diabetic rats without polyphenols consumption

(sciatic nerve) and full (DRG, spinal cord) return of the contents of NT to the control level.

It may be assumed that polyphenol complexes of grape wine partially or completely prevent an increase in the content of nitrotyrosine-modified proteins and this can recover or stabilize the function of retinae and the peripheral nervous system of the body affected by diabetes. The damage caused by peroxynitrite can lead to motor and sensory neuropathies through various mechanisms, including activation of PARP-1 [20]. Thus, in response to DNA damage by reactive oxygen species, the reparatory complex, which includes PARP enzyme poly(ADP-ribosyl)ation over 300 core proteins and some cytoplasmic proteins, is activated. Therefore, the activation of PARP-1 and oxidative/nitrosative stress can be mutually reinforcing, and the determination of their levels is necessary in order to evaluate the degree of tissue damage under conditions of diabetes. The content of poly(ADP-ribosyl)ated proteins recorded in microphotographs of retinae increased by 47 % in rats with diabetes, and this increase was normalized by the consumption of polyphenols (Fig. 12.5).

The intensity of luminescence in immunofluorescent microphotographs, which identified the level of poly(ADP-ribosyl)ated proteins in cells of the sciatic nerve, increased by 51 % in rats with diabetes [11]. This increase was normalized by polyphenol consumption (Fig. 12.6). At the same time, the level of poly(ADP-ribosyl) ated proteins in spinal cord and DRG in control and diabetic rats did not differ significantly (Fig. 12.7).

As was noted above, the abnormal changes in neuropathy occur in and extend from the distal parts of the nerves. Thus, in the diabetic neuropathy, the loss of sensory sensitivity is first to occur. After that, demyelination of nerve conduction with

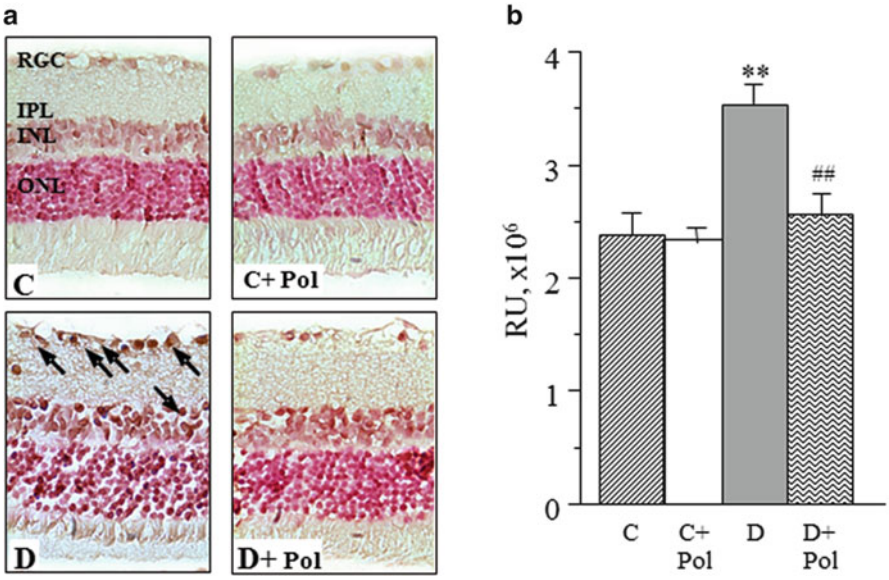

Fig. 12.5 Representative microphotographs of immunofluorescent staining of poly (ADP-ribose) in retine of control and diabetic rats with and without polyphenol consumption. Microphotographs (40×) of immunohistochemical staining of poly(ADP-ribose) in retinae of control and diabetic rats with and without polyphenol consumption (**a**). Total poly(ADP-ribose) content (relative units per image) in retinae (**b**). *Arrows* show examples of poly(ADP-ribosyl)ated proteins of retinae cells. *RGC* retinal ganglion cells, *IPL* inner plexiform layer, *ONL* outer nuclear layer, *INL* inner nuclear layer. Means ± s.e.m., n = 10–15 per group. *C* control rats, *D* diabetic rats, *Pol* polyphenol complexes of grape wine. **p < 0.01 vs. controls, ##p < 0.01 vs. diabetic rats without polyphenol consumption

loss of the sensory and motor nerve velocity takes place. Degeneration both of a single axon and the nerve fiber occurs in the direction from the periphery to the center and is characterized by the term "death back" [6]. It should be noted that our data indicate that PARP-1 is activated in the sciatic nerves, but the activation is absent in the spinal cord and DRG of rats with DM (Fig. 12.7). This confirmed and extended the interpretation of previous studies, which have shown that structurally different inhibitors of PARP-1 restored the conductivity of both motor and sensory nerves, offset the phenomenon of neurovascular dysfunction, restored the energy of the peripheral nervous system and removed signs of sensory neuropathy in rats with 4 weeks of diabetes [20]. Therefore, the activation of PARP-1 in the sciatic nerve and its absence in the spinal cord and DRG indicate that this enzyme occupies a central role in the development of an early neuropathy in the peripheral nervous system during DM [21]. The ability of polyphenols to inhibit PARP-1 supports their potential to prevent the early development of angiopathy during diabetes.

The activity of the enzymatic antioxidant system during DM can change in different ways in different tissues, but always decreases. Consistent with this idea, a

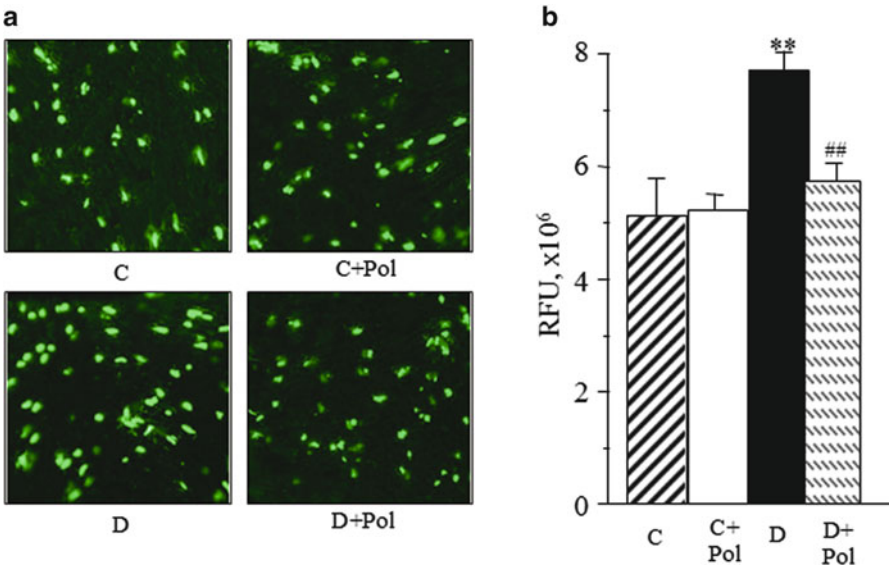

Fig. 12.6 Representative microphotographs (100×) of immunofluorescent staining of poly(ADP-ribose) in sciatic nerves (**a**). Intensity of poly(ADP-ribose) fluorescence (relative fluorescence units per image) in sciatic nerves (**b**). *C* control groups, *D* diabetic groups, *Pol* polyphenol complexes of grape wine. Data are expressed as mean ± s.e.m., n = 5–7. **p < 0.01 vs. controls, ##p < 0.01 vs. diabetic rats without polyphenols consumption

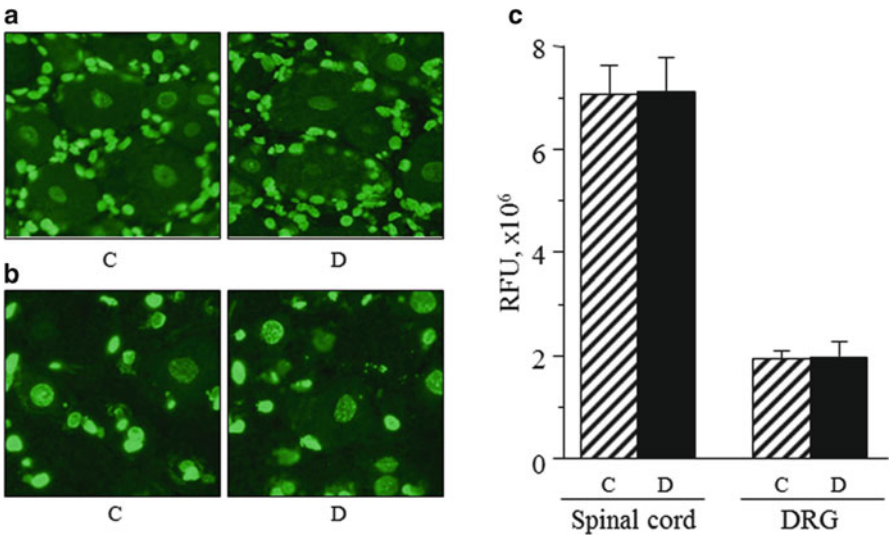

Fig. 12.7 Representative microphotographs (100×) of immunofluorescent staining of poly(ADP-ribose) in spinal cord (**a**) and in DRG (**b**). Intensity of poly(ADP-ribose) fluorescence (relative fluorescence units per image) in DRG and spinal cord (**c**). *C* control groups, *D* diabetic groups, *Pol* polyphenol complexes of grape wine. Data are expressed as mean ± s.e.m., n = 5–7

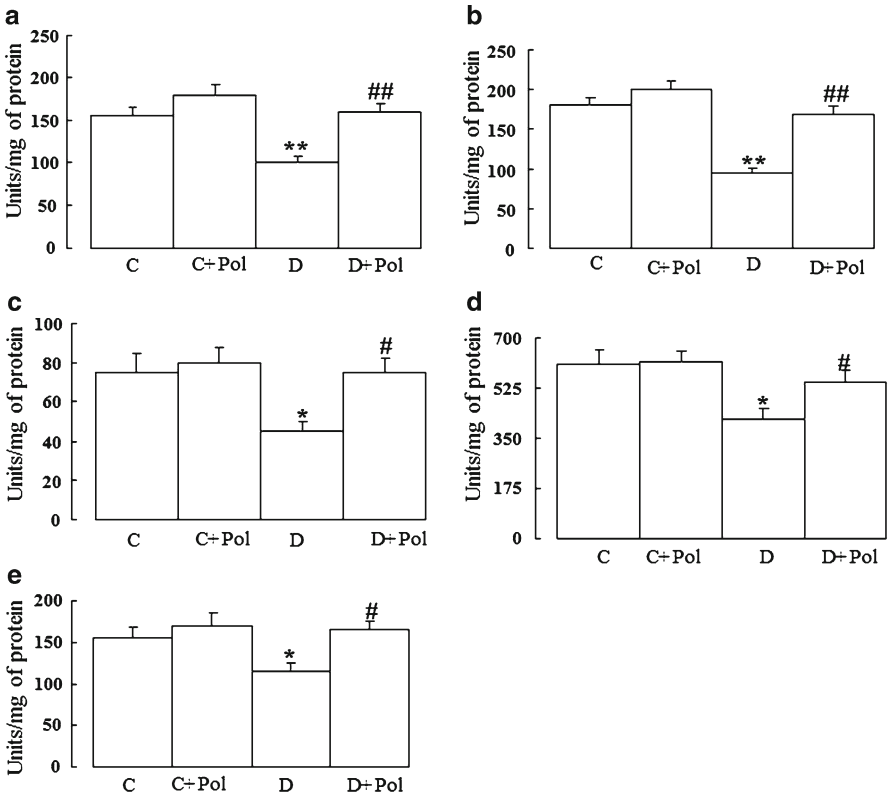

Fig. 12.8 The activity of superoxide dismutase in the kidney (**a**), retina (**b**), sciatic nerve (**c**), spinal cord (**d**) and DRG (**e**). *C* control, *C* + *Pol* control + polyphenol complexes of grape wine, *D* diabetic, *D* + *Pol* diabetic + polyphenols complexes of grape wine. Mean ± s.e.m., n = 5–7. *P < 0.05 and **P < 0.01 vs. controls; #P < 0.05 and ##P < 0.01 vs. untreated diabetic group

significant decrease in the activity of superoxide dismutase was shown in the sciatic nerve, dorsal root ganglia and spinal cord, kidney and retina (by 40, 26, 32, 31 and 47 %, respectively) (Fig. 12.8).

A decrease in the activity of SOD is directly associated with the accumulation of superoxide anions, which is typical for most tissues under DM conditions. Violation of the mechanism of utilization of these anions leads to the formation of another oxidant – peroxynitrite ($ONOO^-$), which can interact with proteins, nitrite them on tyrosine residues and thereby alter their biological properties [24]. Besides peroxynitrite, the formation of products of protonation of nitric oxide (e.g., NO^+, NO^-, NO^{2-}, NO^{3-}) has been reported. These moeties are especially detrimental for enzymes, because they can modify amino acid residues that can ultimately decrease SOD activity. The direct interaction of NO with Cu^{2+} in the active center of SOD causes an inhibition of its activity. It is complemented by the non-enzymatic glycosylation of amino acid residues also affecting SOD activity.

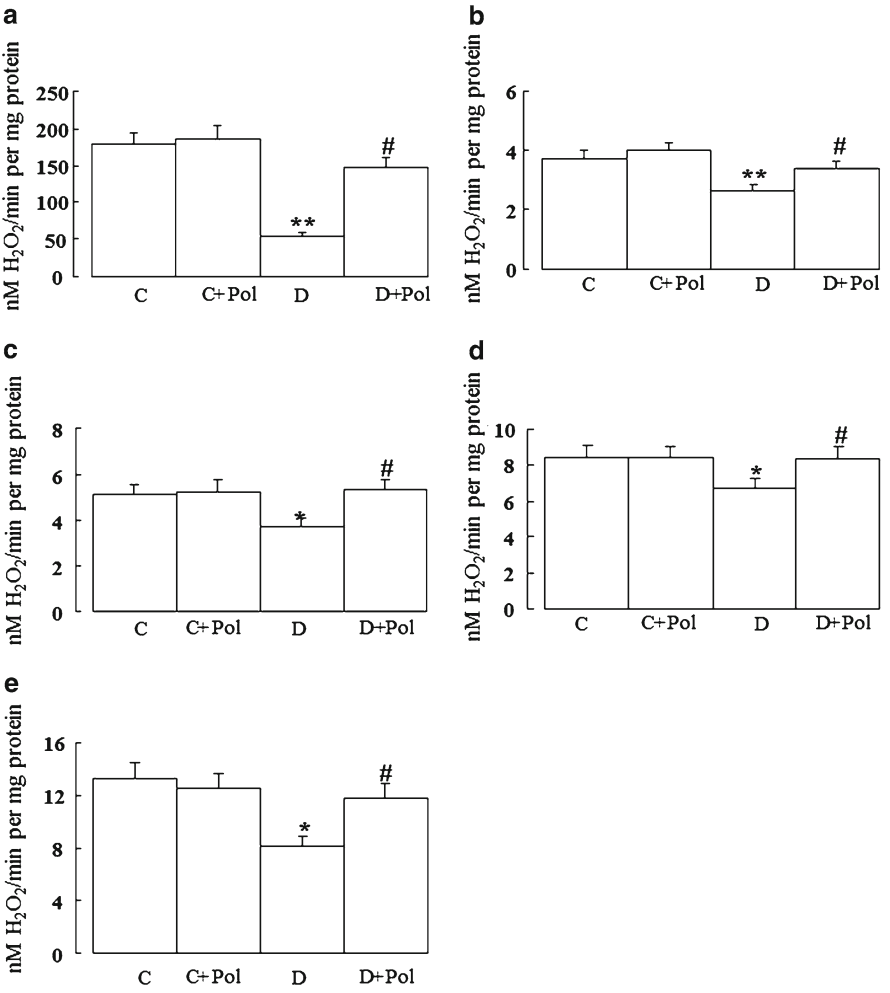

Fig. 12.9 The activity of catalase in the kidney (**a**), retinae (**b**), sciatic nerve (**c**), spinal cord (**d**) and DRG (**e**). *C* control, *C* + *Pol* control + polyphenol complexes of grape wine, *D* diabetic, *D* + *Pol* diabetic + polyphenol complexes of grape wine. Mean ± s.e.m., n = 5–7. *P < 0.05 and **P < 0.01 vs. controls. #P < 0.05 and ##P < 0.01 vs. untreated diabetic rats

During DM, catalase activity is also decreased in the sciatic nerve, DRG, spinal cord, kidney and retina by 27, 37, 21, 70 and 29 %, respectively (Fig. 12.9). A decrease in the activity of this enzyme can be explained by similar mechanisms as for SOD. Additionally, nitric oxide can directly come into contact with iron-porphyrine complexes of catalase, forming nitric derivatives. The appearance of heme-NO complexes prevents binding of H_2O_2 to the active center of catalase, and, hence, its expansion. Nitrite ions are also able to directly communicate with the iron component of the heme part of the enzyme, decreasing its activity [17].

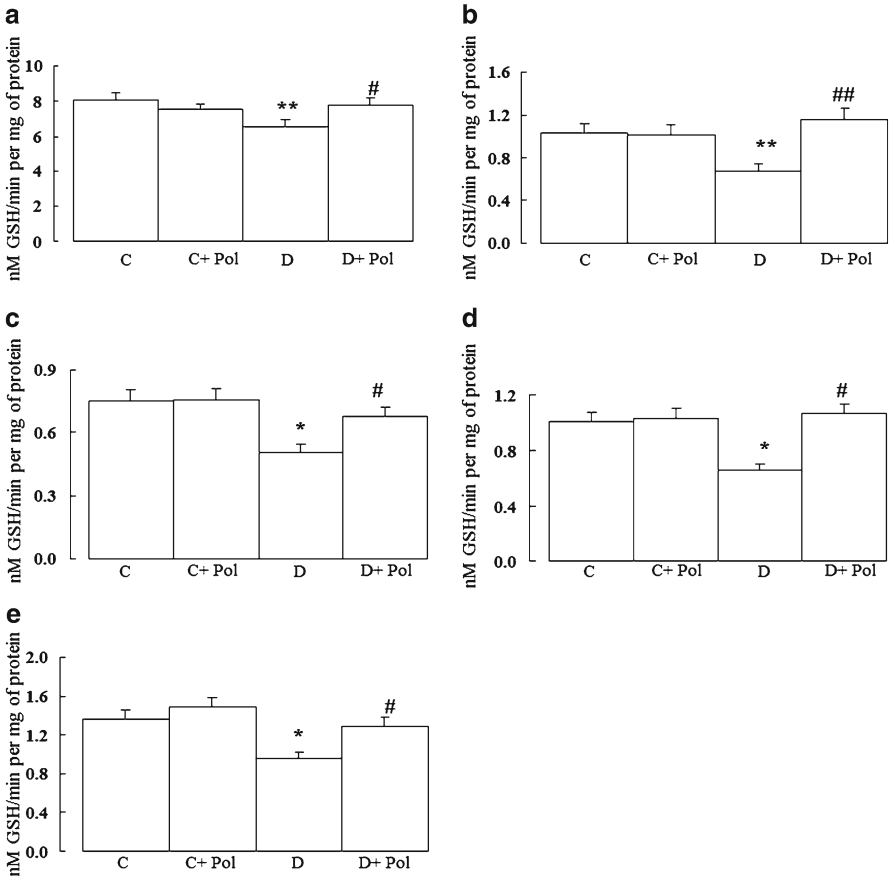

Fig. 12.10 The activity of glutathione peroxidase in the kidney (**a**), retinae (**b**), sciatic nerve (**c**), spinal cord (**d**) and DRG (**e**). *C* control, *C* + *Pol* control + polyphenol complexes of grape wine, *D* diabetic, *D* + *Pol* diabetic + polyphenol complexes of grape wine. Mean ± s.e.m., n = 5–7. *P < 0.05 and **P < 0.01 vs. controls; #P < 0.05 and ##P < 0.01 vs. untreated diabetic group

The glutathione system (GS) is especially important under oxidative-nitrosative stress (ONS). It provides effective protection to the cells against the effects of ONS and, therefore, when it is depleted, serious consequences for the organism can occur. Apart from CAT, the neutralization of hydrogen peroxide is also curried out by GPO. The affinity of GPO for H_2O_2 is significantly higher than for catalase.

During DM, the activity of GPO in the sciatic nerve, DRG and spinal cord, kidney and retina was reduced by 33, 30, 35, 19, 33 %, respectively, and the one of glutathione reductase (GR) was reduced by 37, 15, 30, 30 and 29 %, respectively (Figs. 12.10 and 12.11).

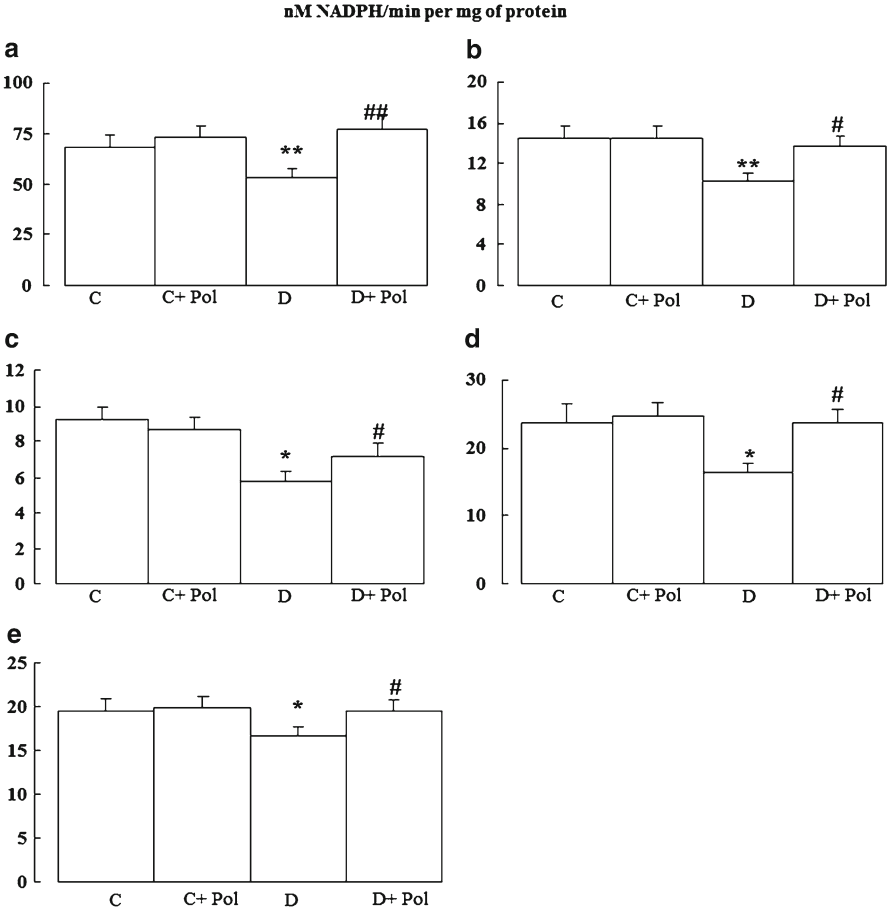

Fig. 12.11 The activity of glutathione reductase in the kidney (**a**), retinae (**b**), sciatic nerve (**c**), spinal cord (**d**) and DRG (**e**). *C* control, *C* + *Pol* control + polyphenol complexes of grape wine, *D* diabetic, *D* + *Pol* diabetic + polyphenol complexes of grape wine. Mean ± s.e.m., n = 5–7. *P < 0.05 and **P < 0.01 vs. controls; #P < 0.05 and ##P < 0.01 vs. untreated diabetic group

GPO activity depends on the content of reduced glutathione, the level of which is supported by the intracellular concentration of GR. GR activity is in turn determined by the level of reduced nicotinamide coenzymes.

A deficiency in energy substrates having a directly proportional effect on the efficiency of immune systems is taking place during DM. There is no effective protection without substrates with enough energy.

After consumption of the polyphenol complex, the activity of these enzymes was completely or partially restored to control values. The level of the

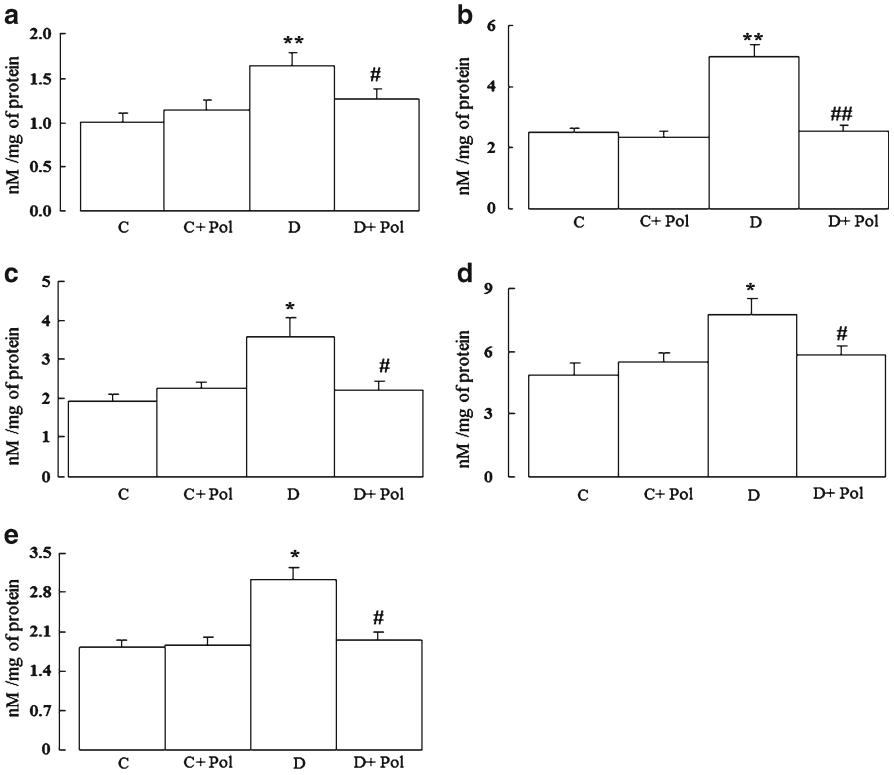

Fig. 12.12 The level of the sulfocarbanilide-positive content of LPO products in the kidney (**a**), retinae (**b**), sciatic nerve (**c**), spinal cord (**d**) and DRG (**e**). *C* control, *C* + *Pol* control + polyphenol complexes of grape wine, *D* diabetic, *D* + *Pol* diabetic + polyphenol complexes of grape wine. Mean ± s.e.m., n = 5–7. *P < 0.05 and **P < 0.01 vs. controls; #P < 0.05 and ##P < 0.01 vs. untreated diabetic group

sulfocarbanilide-positive content of LPO products in the kidney, retinae, sciatic nerve, spinal cord and DRG was also restored to control values (Fig. 12.12).

Thus, the natural polyphenol complex of grape wine has a significant anti-diabetic effect at the level of the whole organism. It protected it from dehydration and increased the activity of the antioxidant enzyme system in the sciatic nerve, DRG, spinal cord, kidney and retina. The levels of nitrosilative and PARylated proteins were almost restored to the control levels. The biochemical mechanisms of action of the natural polyphenol complex of grape wine are the subject of further research, but this natural complex can certainly be used in the treatment of complications of diabetes and the development of new anti-diabetic drugs.

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Chapter 13
Mitochondrial BK_{Ca} Channel as a Target for Cardioprotection

František Kolář

Abstract Large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are widely expressed in the plasma membrane of various types of cells and play important roles in many physiological processes by providing negative feedback for membrane depolarization and Ca^{2+} entry. Their mitochondrial counterparts located in the inner membrane are thought to be involved in the control of mitochondrial functions and serve as mediators of cytoprotection. This review briefly outlines basic knowledge of the molecular structure, sources of tissue diversity, and modulation of BK_{Ca} channels by endogenous and pharmacological agents. Particular attention is paid to the heart with an emphasis on the role mitochondrial BK_{Ca} channels in various forms of cardioprotection against acute ischemia/reperfusion injury.

13.1 Introduction

Large-conductance Ca^{2+}-activated K^+ channels ($K_{Ca}1.1$), also termed Big-K (BK, BK_{Ca}) or Maxi-K channels because of their unusually high unitary conductance (~100–300 pS), are almost ubiquitously expressed in the plasma membrane of a variety of cell types, including excitable and non-excitable cells. Unique among ion channels, BK_{Ca} channels are synergically activated in response to depolarizing membrane potentials and increased cytosolic concentration of Ca^{2+} [46], thus serving as negative feedback regulators for events that lead to membrane depolarization and Ca^{2+} entry. This dual control is characteristic for BK_{Ca} channels, allowing them to

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couple Ca^{2+}-dependent intracellular signaling to membrane potential and play important roles in a number of physiological processes such as, for example, regulation of smooth muscle cell tone, endothelial function, neuronal excitability and synaptic transmission, hormone secretion, hearing, or immune responses etc. [9, 22, 59 and references therein]. Dysfunction of BK_{Ca} channels can, therefore, contribute to pathogenesis of a number of diseases including hypertension, ischemic heart disease, asthma, various neurological disorders, urinary problems, hearing loss, radiation injury etc. In view of their multiple pathophysiological roles, BK_{Ca} channels have been considered a promising target for pharmacotherapy and a number of BK_{Ca}-modulating agents have been designed and synthesized [52]. Research interest in BK_{Ca} channels has been further stimulated by their discovery in mitochondria [69] and, in particular, by the observation that activation of these mitochondrially located channels (mBK_{Ca}) can protect the heart against lethal injury caused by acute ischemia/reperfusion (I/R) [81].

13.2 Structure and Diversity of BK_{Ca} Channels

The functional BK_{Ca} channel is a tetrameric assembly of pore-forming α-subunits (Slo1), which are sufficient for channel activity. This subunit is encoded by a single gene (*slowpoke*, *slo*/*slo1*, *KCNMA1*), first cloned from mutant fruit fly [3]. Similar to voltage-dependent K^+ (Kv) channels comprising six transmembrane segments, the BK_{Ca} channel voltage sensing domain is formed by segments S1-S4 and the pore-gate domain by segments S5-S6 [50]. An additional S0 segment, unique to BK_{Ca} channels, confers the NH_2 terminus to the extracellular side and is required for modulation by β-subunit [76]. The large intracellular COOH terminus contains several alternative splicing sites and four hydrophobic segments regulating K^+ conductance and containing two putative high affinity Ca^{2+} binding sites. Detailed description of the BK_{Ca} channel structure can be found elsewhere [22, 36, 59].

Although the α-subunit is encoded by only one gene, the properties of BK_{Ca} channels are considerably diverse depending on the cell type and other conditions. One of the sources of this diversity is alternative splicing, which creates various transcripts translated into channel proteins with different kinetics, voltage dependence and Ca^{2+}-sensitivity. Association of the channel with auxiliary β-subunits is another source of BK_{Ca} diversity, obviously more important than alternative splicing.

Auxiliary β-subunits are formed by two transmembrane segments connected by a large extracellular loop and flanked by short NH_2 and COOH termini oriented intracellularly. Four β-subunits (β_1-β_4) have been found so far in various mammalian tissues, encoded by genes *KCNMB1-4*. The assembly of various β-subunits with α-subunit splice variants produces large variety of BK_{Ca} channels with markedly different biophysical properties, sensitivity to endogenous modulators and pharmacological agents, and tissue distribution, serving to distinct physiological functions [9, 30, 59, 62 and references therein].

The first identified BK_{Ca} β-subunit was β_1, which is primarily expressed in smooth muscle cells [42]. BK_{Ca} β_2- and β_3-subunits have been found in various types of cells and tissues [9], while β_4-subunit is predominantly expressed in neuronal tissue [15]. The BK_{Ca} channel lacking β-subunits has low sensitivity to Ca^{2+} and its opening occurs at very positive values of membrane potential [21]. Binding of the β_1-subunit highly increases Ca^{2+} and voltage sensitivity, slows deactivation kinetics [62], and mediates effects of a number of endogenous modulators such as, for example, 17β-estradiol [75]. The β_4-subunit slows activation and deactivation kinetics, and has opposite effects on Ca^{2+} sensitivity at low (decreasing) and high (increasing) Ca^{2+} concentrations [15, 32]. In addition, various β-subunits exhibit distinct effects on the expression of the BK_{Ca} α-subunit, trafficking of the channel to the cell membrane and, importantly, its pharmacological properties [9]. For example, while the β_1-subunit confers high sensitivity of the channel to peptide toxins such as charybdotoxin or iberiotoxin (see Sect. 13.3.2), co-assembly with the β_4-subunit makes the channel practically insensitive to these blockers [51].

13.3 Modulation of BK_{Ca} Channel Activity

In addition to membrane voltage and cytosolic Ca^{2+} concentration, functional properties of assembled BK_{Ca} channels can be significantly modulated by posttranslational mechanisms such as phosphorylation [67] or *N*-glycosylation [33], as well as by numerous endogenous molecules, naturally occurring compounds and synthetic agents [36, 52]. Moreover, BK_{Ca} channels physically associate with other proteins to form large functional complexes. For example, tight association of BK_{Ca} channels with various types of Ca^{2+} channels [9, 45] enables efficient feedback control of local Ca^{2+} influx.

13.3.1 Posttranslational Modifications

Several common serine/threonine protein kinases potently affect the BK_{Ca} channel activity by phosphorylation of various sites at the COOH terminus of the α-subunit. Phosphorylation can either stimulate or inhibit the channel depending on its composition, tissue localization and protein kinase involved, making this issue extremely complex and not fully understood. For example, both cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase activate BK_{Ca} channels in smooth muscle cells by increasing their Ca^{2+} and voltage sensitivity, while protein kinase C (PKC) usually has inhibitory effects [44, 67]. Thus, PKA and PKG activated by hormones or other stimuli contribute to relaxation of smooth muscle via BK_{Ca} opening, in opposite to PKC-mediated phosphorylation, which promotes contraction. Interestingly, the phosphorylation of BK_{Ca} channel by PKC not only

inhibits its activity, but also abolishes the stimulatory effects of PKA and PKG, thereby preventing muscle response to relaxing factors [83]. In contrast, the activation of smooth muscle BK_{Ca} channels by PKC has also been reported, but this effect is likely indirect, mediated by PKG-dependent phosphorylation [4]. Neuronal BK_{Ca} channels, however, can be directly phosphorylated and activated by PKC [41].

Extracellular domain of the β-subunit exists in *N*-glycosylated form [13, 33] and glycosylation represents another posttranslational mechanism, which can modulate BK_{Ca} activity. Hagen and Sanders [33] demonstrated that glycosylation of the β_1-subunit modified biophysical properties of BK_{Ca} channels in smooth muscle cells and inhibited their opening. In these experiments, enzymatic deglycosylation stimulated the channel activity by increasing its open probability and mean open time.

13.3.2 BK_{Ca} Channel Modulators

A growing number of endogenous substances have been reported to directly or indirectly modulate BK_{Ca} channels. They include ions such as H^+ and Mg^{2+}, reactive oxygen species (ROS), nitric oxide, carbon monoxide, heme, arachidonic acid and its metabolic products, phospholipids, ceramide, cholesterol, steroid hormones such as estradiol, testosterone and glucocorticoids, anandamide, prostacycline and many others. Detailed description of mechanisms underlying effects of these molecules on BK_{Ca} activity exceeds the scope of this chapter and can be found in recent reviews [29, 36, 52, 80].

Countless natural and synthetic agents have been tested in order to find out potent and sufficiently selective BK_{Ca} modulators, but none of them has been approved for clinical use until now. Nardi and Olesen [52] provided a comprehensive overview of many agents with potential BK_{Ca}-modulating properties. Here we mention only those openers, inhibitors and blockers, which have been used recently as useful pharmacological tools in experimental research of BK_{Ca} channels and their physiological roles. It should be mentioned, however, that none of the agents available appears to be strictly specific to BK_{Ca} channels [28, 74].

Charybdotoxin and iberiotoxin, peptides isolated from venom of scorpions, are the most common potent BK_{Ca} channel blockers, which irreversibly bind to the pore and block its conduction pathway [17]. Iberiotoxin is more selective than charybdotoxin, which affects other types of K^+ channels in addition to blocking BK_{Ca} [60]. As mentioned above, the assembly of the channel α-subunit with distinct regulatory β-subunits markedly affects its sensitivity to these toxins [51]. Mycotoxin paxilline has been used in experiments probably most often as a potent reversible BK_{Ca} channel inhibitor with high selectivity. Nevertheless, its potential side-effects, such as the inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase [10] or inositol 1,4,5-trisphosphate receptors [47] cannot be excluded at concentrations higher than 5 μM.

Among BK_{Ca} openers, the synthetic benzoimidazolone compound NS1619 [58] has received undoubtedly the highest attention as a conventional pharmacological

tool targeting this channel, although more recent studies pointed to its rather low potency and selectivity [9]. It has been shown that NS1619 exhibits some BK_{Ca}-independent effects at higher concentrations, such as the inhibition of L-type Ca^{2+} channels [61]. In search for better pharmacological tools, the novel compound NS11021 was introduced recently as structurally different, more potent and more specific BK_{Ca} opener when compared to NS1619 [7]. It increases the open probability of the channel without affecting its conductance. Derivatives of dehydroabietic acid diCl-DHAA and Cym04 are other novel potent BK_{Ca} openers [64]. Interestingly, Cym04 and NS1619 do not affect splice variant Slo1_9a of the channel, which is expressed primarily in the neuronal tissue [72]. Obviously, further development of tissue- and function-specific BK_{Ca} openers is needed [29].

13.4 Mitochondrial BK_{Ca} Channels

Since the first identification of BK_{Ca} channels in the inner mitochondrial membrane of human glioma cell line [69], many additional studies documented their mitochondrial localization in other types of cells. The presence of functional mBK_{Ca} channels was directly observed by patch-clamp recordings of single-channel activity sensitive to BK_{Ca} blockers in mitoplasts isolated, for example, from ventricular myocytes [57, 81], skeletal muscle [71], liver and astrocytes [20]. Consistent with these findings, the pore-forming BK_{Ca} α-subunits were detected in mitochondria by immunohistochemistry and Western blotting [24, 63, 68, 81]. The distribution of different β-subunits localized to mitochondria appears to be tissue-specific. The β_4-subunit was detected in mitochondria from skeletal muscle [71], H9c2 cells [24] and brain tissue [70], whereas the β_1-subunit seems to predominate in cardiac mitochondria [5, 13, 57, 77]. Taking together, the evidence in favor of the mitochondrial presence of functional BK_{Ca} channel and its constituents seems more convincing than the existence of the mitochondrial ATP-sensitive K^+ channel (mK_{ATP}), which is a matter of continuing controversy [27]. Although functional characteristics and pharmacological sensitivity of mBK_{Ca} channel resemble those of its counterpart located in the plasma membrane [81], its exact molecular identity is unknown. Recently, a unique isoform of BK_{Ca} channel α-subunit was found as a single predominant α-isoform expressed in mouse cardiomyocytes [43]. It may represent mBK_{Ca} subunit, as sarcolemmal BK_{Ca} channels have not been cloned from ventricular myocytes.

In contrast to plasma membrane BK_{Ca} channels, which by promoting K^+ efflux from a cell counteract membrane depolarization, the opening of mBK_{Ca} channels leads to K^+ influx into the matrix, partially depolarizing the inner membrane. Because the inner membrane potential is the driving force for Ca^{2+} influx into the matrix by Ca^{2+} uniporter, mBK_{Ca} channels may exert negative feedback preventing mitochondrial Ca^{2+} overload [39]. In addition, they are considered to play important roles in controlling matrix volume and mitochondrial energetics [2], ROS formation [34, 35], permeability transition [20], oxygen (hypoxia) sensing [31], and other physiological processes.

13.5 BK_{Ca} Channels in the Heart

In contrast to other cell types, neither the functional BK_{Ca} channel nor its constituents are known to be expressed in the sarcolemma of ventricular myocytes [63]. BK_{Ca} subunits detected in ventricular myocytes as well as BK_{Ca}-dependent effects observed in these cells can be, therefore, most likely attributed to mBK_{Ca} channels. However, the heart contains also other types of cells, which express plasma membrane BK_{Ca} channels, such as coronary smooth muscle cells [11, 14], coronary endothelial cells [80], endocardial endothelium [37] or intracardiac autonomic neurons [23]. These channels influence heart functions under physiological conditions and may contribute to dysfunction in various disease states. For example, BK_{Ca} channels of smooth muscle have been implicated in coronary vasorelaxation during exercise [14] or myocardial ischemia [55]. Moreover, impaired activity of BK_{Ca} channels contributes to coronary vascular dysfunction in aged rats [53] as well as in various experimental models of diabetes, dyslipidemia and metabolic syndrome [11, 48, 82].

A recent study proposed that BK_{Ca} channels play a role in the regulation of heart rate, based on the observation that paxilline caused bradycardia and this effect was absent in mice with a deletion in the gene encoding for the BK_{Ca} channel α-subunit [38]. The cellular location of channels responsible for this effect is unknown, as contributions of vascular, neuronal or mitochondrial BK_{Ca} channels seems unlikely. Interestingly, the existence of sarcolemmal BK_{Ca} channels in cow cardiac Purkinje fibers was reported in a single study [16], but we are not aware of any study examining the expression of this channel in primary pacemaker cells.

13.6 Role of mBK_{Ca} Channels in Cardioprotection

There is a general consensus that mitochondria are key players in various forms of both innate and acquired cardioprotection against injury caused by acute I/R insult. K^+ influx into the matrix mediated by K^+ channels of the inner membrane has been considered as an important step in myocardial salvation. Besides putative mK_{ATP} channels, which have received high attention in this context, activation of mBK_{Ca} channels recently emerged as an important alternative mechanism for mitochondrial K^+ uptake and cell survival.

13.6.1 Cardioprotection Induced by BK_{Ca} Openers

Xu et al. [81] were the first to demonstrate the infarct size limiting effect of the BK_{Ca} opener NS1619 and the complete blockade of protection by the inhibitor paxilline. This observation was confirmed by a number of subsequent studies using various experimental models and endpoints of I/R injury (Table 13.1). Conclusions on the

Table 13.1 Cardioprotective effects of BK_{Ca} openers

Experimental model	Primary endpoint of injury	Protective agent	Protection blocked	Refs.
Guinea pig perfused heart	Infarct size	NS1619	Pax	[81]
Rat perfused heart	Infarct size	NS1619	Pax	[19]
Rat ventricular myocytes	Cell death	NS1619	Pax	[13, 19]
Guinea pig vent. myocytes	Cell death	NS1619	Pax	[66]
Mouse perfused heart	Infarct size	NS1619	Pax	[78]
		NS1619 PC		
Mouse heart in vivo	Infarct size	NS1619 PC	IbTx	[63]
Guinea pig perfused heart	Infarct size	NS1619 PC	Pax	[73]
H9c2 cell line	LDH release	NS1619 PC	Pax, IbTx	[24]
H9c2 cell line	LDH release	NS1619 PoC	Pax, IbTx	[25]
Rat perfused heart	Infarct size	NS11021	Pax	[8]
		NS11021 PoC		
Rat ventricular myocytes	Cell death	NS11021	Pax	[12]
		NS11021 PoC		
Rat ventricular myocytes	Cell death	diCl-DHAA	Pax	[65]
Rabbit perfused heart	Infarct size	Cilostazol	Pax	[26]

diCl-DHAA 12,14-dichlorodehydroabietic acid, *PC* preconditioning, *PoC* postconditioning, *Pax* paxilline, *IbTx* iberiotoxin

involvement of mBK_{Ca} in cardioprotection are based on pharmacological evidence in all these studies: most of them relied on the use of NS1619 and paxilline. However, the effects of NS1619 should be interpreted with caution due to rather low specificity of this agent, which can potentially affect cardiac ischemic tolerance by mBK_{Ca}-independent effects. It has been shown to directly inhibit several sarcolemmal ion channels [8] and to promote potassium influx into the matrix not solely through BK_{Ca} channels, but also through nonspecific ion transport mechanism [1]. However, the blockade of NS1619-induced protection by paxilline seems to support the involvement of mBK_{Ca} channels, as no major concerns have been raised with respect to this inhibitor used at low concentrations [84].

The novel BK_{Ca} opener NS11021 with higher potency and better selectivity compared with NS1619 [7] was shown to reduce infarct size and improve postischemic recovery of contractility in isolated rat hearts [8]. Using isolated ventricular myocytes subjected to simulated I/R (metabolic inhibition and reenergization), we observed paxilline-sensitive cytoprotective effects of NS11021 at submicromolar concentrations [12]. Interestingly, both NS1619 and NS11021 exhibited protective effects when used as preconditioning stimuli [24, 63, 73] or when applied only at reperfusion (postconditioning) [8, 12, 26]. Moreover, NS13558 obtained by methylation of the terminal tetrazolic ring of NS11021 without affecting its overall structural conformation [6] retains comparable biological activity towards other ion channels as NS11021 at higher concentrations, but it lacks BK_{Ca} activator properties and is not cardioprotective [6, 12], consistent with the absence of stimulatory effect on mitochondrial K^+ uptake and matrix volume [2]. These results strongly suggest that the protective effects of NS11021 can be ascribed to the opening of mBK_{Ca} channels.

Table 13.2 Involvement of mBK_{Ca} channels in cardioprotection by other stimuli

Experimental model	Primary endpoint of injury	Protective agent	Protection blocked	Ref.
Rat ventricular myocytes	Cell death	17β-estradiol	Pax	[57]
Mouse perfused heart	Infarct size	Sildenafil	Pax	[77]
Rabbit perfused heart	Infarct size	Adrenomedullin	Pax	[54]
Rat perfused heart	Infarct size	U50,488H	Pax	[18]
Rat ventricular myocytes	Cell death			
Mouse perfused heart	Contractile dysfunction	EETs	Pax	[40]
Rabbit perfused heart	Infarct size	Anandamide	Pax	[56]
Rat perfused heart	Infarct size	Ischemic PC	Pax	[19]
Rat ventricular myocytes	Cell death			
Mouse heart in vivo	Infarct size	Desflurane PC	IbTx	[63]
H9c2 cell line	LDH release	CPA PC	Pax, IbTx	[24]
H9c2 cell line	LDH release	CPA PoC	Pax, IbTx	[25]
Rat ventricular myocytes	Cell death	Chronic hypoxia	Pax	[13]

U50,488H: κ-opioid receptor agonist; *CPA* adenosine A_1 receptor agonist N^6-cyclopentyladenosine, *EETs* epoxyeicosatrienoic acids; *PC* preconditioning, *PoC* postconditioning, *Pax* paxilline, *IbTx* iberiotoxin

Direct activation of mBK_{Ca} channels is considered to underlie also the cytoprotective effect of diCl-DHAA [65] and infarct size-limitation by the phosphodiesterase type 3 inhibitor cilostazol [26].

13.6.2 Role of mBK_{Ca} Channels in Cardioprotection by Other Stimuli

Several studies listed in Table 13.2 indicated that mBK_{Ca} channels are involved in both classic and delayed ischemic preconditioning [19, 78] and pharmacological preconditioning induced by adenosine A_1 receptor agonist N^6-cyclopentyladenosine [24]. This agent also protected rat embryonic cardiomyoblast-derived H9c2 cells by postconditioning in a manner dependent on mBK_{Ca} channel opening [25]. Concerning anesthetic preconditioning, the available data are rather controversial. While Redel et al. [63] demonstrated that desflurane-induced myocardial preconditioning in mice was mediated in part by activation of mBK_{Ca} channels, another recent study indicated that these channels are not necessary for preconditioning by isoflurane, as its protective effect was preserved in *Slo1*$^{-/-}$ knockout mice [79]. Instead, opening of Ca^{2+}-insensitive mBK channels encoded by another gene *Slo2* seems to be responsible for myocardial protection by isoflurane shown in the later report.

It has been demonstrated that PKA-dependent activation of mBK_{Ca} channels play a role in cardioprotective mechanisms conferred by sildenafil [77] and adrenomedullin [54]. Furthermore, these channels are involved in protection by 17β-estradiol [57], κ-opioid receptor agonist [18], epoxyeicosatrienoic acids [40], or canabinoid receptor

agonist anandamide [56]. It appears that at least some of these effects require the presence of the BK_{Ca} β_1-subunit. For example, the activation of the channel and the improved ischemic tolerance of ventricular myocytes induced by 17β-estradiol resulted from its functional interaction with the β_1-subunit [57], and selective knockdown of the β_1-subunit with siRNA blunted the delayed infarct size-limiting effect of sildenafil [77]. No de novo synthesis of the β_1-subunit was detected after sildenafil treatment in the later study, suggesting that protection was dependent on activation of existing β_1-subunits rather than its upregulation.

In addition, we have shown recently that mBK_{Ca} channels significantly contribute to the long-lasting cardioprotection induced by adaptation to chronic hypoxia [13]. In contrast to the inhibitory effect of hypoxia on the activity of plasma membrane BK_{Ca} channels, the opposite response of mBK_{Ca} channels has been reported [20, 31]. Oxygen tension regulates also the expression BK_{Ca} β_1-subunit in cardiomyocytes in a manner dependent on hypoxia-inducible factor-2α [5]. However, this effect may be only transient, as we did not find any change in protein level of the β_1-subunit in rat ventricular myocytes after long-lasting hypoxic exposure. Interestingly, chronic hypoxia caused marked deglycosylation of the β_1-subunit [13] and this modification can potentially activate the channel, as demonstrated in smooth muscle cells [33]. Nevertheless, it seems unlikely that this mechanism is responsible for cytoprotection by chronic hypoxia, because another non-protective model of hypoxia led to even more pronounced deglycosylation (unpublished).

13.6.3 Mechanism of Protection by mBK_{Ca} Opening

The cardioprotective mechanism downstream of mBK_{Ca} opening remains not fully understood, analogous to that of mK_{ATP} channels. It has been attributed to increased matrix K^+ uptake and volume, improved respiratory control [2], attenuation of mitochondrial Ca^{2+} overload [39, 78], prevention of permeability transition [18–20], and inhibition of caspase-3 activation and apoptosis [24]. It appears, however, that the protective pathway is more complex, likely involving ROS signaling and activation of redox-sensitive protein kinases.

Studies on mitochondrial ROS formation in response to BK_{Ca} opening are controversial. For example, either increased or decreased generation of superoxide by cardiac mitochondria treated with NS1619 was reported, depending on substrate and energetic conditions [34, 35]. It has been proposed that BK_{Ca} opening may inhibit complex I-dependent ROS production in the matrix and stimulate complex III-dependent formation of superoxide, which is responsible for the protective signal transduction to the cytosol [49]. Indeed, Stowe et al. [73] showed that myocardial preconditioning by NS1619 depends on ROS signaling, as the bracketing of the opener with a superoxide dismutator antagonized its protective effect. Similarly, superoxide dismutase mimetic tempol also abolished the NS11021-induced cytoprotection in our experiments [12]. Consistent with these observations, our preliminary data showed that NS11021 (but not its inactive analogue NS13558)

added to energized myocytes slightly but significantly increased ROS formation, and paxilline or tempol blocked this effect in agreement with blunting cytoprotection (unpublished results). These results suggest that ROS signal occurred downstream of BK_{Ca} opening and not as a consequence of any potential side-effect of NS11021.

In conclusion, mBK_{Ca} channels may represent novel promising target for pharmacological and other interventions aiming to improve myocardial resistance to acute I/R injury. Obviously, further studies are needed to reveal mBK_{Ca} molecular identity and to elucidate the precise mechanism underlying cardioprotective effects of mBK_{Ca} opening.

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Chapter 14
Protection of Subjects Participating in Clinical Trials

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Abstract Clinical trials constitute one of the final stages along the testing continuum that is so needed for the introduction of new products, methods, and groundbreaking technologies. Currently, most economies have recognized the importance clinical trials play as part of the entire value chain from idea to product. While most jurisdictions have developed systems of safeguards for the protection of human subjects involved in clinical trials, there are huge disconnects among jurisdictions, institutions and investigators. This article provides some generally accepted international standards and guidelines in relation to subject protection as it pertains to recruitment, confidentiality, monitoring, data storage and data transfer. The need for all trial protocols to be reviewed by a qualified and registered ethics committee, as well as that all legal requirements are met to ensure patient protection, is also highlighted. Since governments and industry see clinical trials as a critical and necessary step in the product development process, there is a need for more translational research, which will increase the demand for more participation in clinical trials. This article will therefore, also address the importance of patient protection if we are to meet these requirements.

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14.1 Introduction

Clinical trials constitute one of the final stages along the testing continuum that is so needed for the introduction of new products, methods, and groundbreaking technologies. These experiments using human volunteers are designed to determine whether the investigational product is safe and effective. The designs of clinical trials are typically conducted in stages where smaller safety studies involving healthy volunteers are initially planned, proceeded by larger scale studies involving numerous study subjects. Regardless of the phase of clinical trial, the governing health authority must grant approval of the study in addition to ethical approval by an expert committee before the trial can commence. These guidelines are in place in order to protect the safety and well being of the subjects participating in the clinical trial.

Adopted by the World Medical Association in June 1964, The Declaration of Helsinki outlined the concept of independent review of research protocols. The policy has been amended eight times since, most recently in October 2008 [1]. In 1997, the International Conference on Harmonization provided a guidance document on Good Clinical Practice (GCP) an international ethical and scientific quality standard for clinical trials [2]. This document is used by governments as guidance to regulate clinical trials involving humans. Compliance with GCP ensures the rights and safety of trial subjects are protected, consistent with the principles in the Declaration of Helsinki. In addition, there is also a general understanding that certain forms of risk are unacceptable to impose on human subjects. According to the Nuremberg Code, "No experiment should be conducted where there is an *a priori* reason to believe that death or disabling injury will occur" [3, 4].

In 1979, the USA released a report titled the Belmont Report [5] to outline principles that should be followed for the protection of human subjects in research. This is the cornerstone document of the ethical principles and US regulations for the protection of research participants based on respect for persons, beneficence and justice. Currently, all federal departments and agencies supporting, conducting, or regulating research on humans have agreed to a set of ethical principles and regulations called the Common Rule. The Common Rule is in place to ensure that all of an institution's human research activities are guided by the ethical principles found in the Belmont Report. In Canada, the Tri-Council Policy Statement (TCPS) was first implemented in 1998 and has since been updated in 2010 to TCPS 2 [6]. This policy was formed with reference to leading international ethics policies, including The Declaration of Helsinki. With respect to India, clinical trials are governed by Schedule Y in the Drugs and Cosmetics Act [7], however; the competence of ethics committees in patient protection in clinical research has recently been questioned [8].

There is considerable variation in ethical and moral values between different countries and even disparities exist between different cultures within the same country. While ethics committees/institutional review boards as well as government regulatory bodies are official arbiters of ethical issues, the investigator should be well aware of any ethical issues that the design of the clinical trial may present.

This is particularly relevant when the protocol is for another country, social class or group, other than that of the person, who has developed the protocol [9]. Clinical trial investigators should be guided by ethical principles and patient protection should be a top priority [10]. This does not appear to be the case in some developing countries [11] including India [10, 12]. Quality of clinical trials depends not only on data integrity, but also on subject protection. With globalization, outsourcing and increasing complexities of clinical trials achieving global quality has become challenging [13].

Longstanding ethical principles require that risks clinical trial participants should be minimized and justified by the value of the data that the study is expected to produce [1, 2]. In a 2009 report by the European Parliament [14], the most common ethical violations in developing countries included: (i) clinical trial subjects were not adequately informed about the trial, the risks involved, and in some cases not being advised of their participation in a clinical trial at all; (ii) no guarantee to trial subjects of continuing treatment at the end of the trial; (iii) no local ethics approval of the protocol and (iv) experimental drug being tested against placebo rather than current approved intervention thus exposing patients to additional risks. Accordingly, this article describes some of the generally accepted international standards and guidelines in relation to subject protection in the conduct of clinical trials. We also describe the role of institutional ethics boards and the responsibility of the investigator through the design of the protocol. Attention is also paid to the importance of handling of data and personal information as well as the role of the monitoring process in ensuring patient protection during clinical trials. In addition, reference is also made to clinical trial registration and early termination of trials and how these aspects can contribute to protection of subjects participating in clinical trials.

14.2 Role of Institutional Review Boards in Protecting Subjects Against Unethical Practices

The ethics of clinical trials have been the subject of numerous publications and mandates that are used by institutional review boards on a daily basis. Institutional Review Boards (IRB) or Research Ethics Boards (REB) is a committee comprised of at least five members of various professional backgrounds who assemble to assess the research proposals for scientific content as well as to represent the best interest of the clinical trial participants. The committee is mandated by their institution to evaluate the proposed study for foreseeable risks and benefits, ethical implications and study design. Due to the nature of research as an investigation into the unknown, the likelihood of risk to the participant will be present. The type of risk that a human subject may be exposed to include: physical harm, psychological and/or social harm. Physical harm to a human subject include: injury from the use of equipment involved in the study, side-effect from the experimental treatments or allergic reactions [6]. When participating in a clinical trial a participant may

experience stress if the study involves answering questions that trigger recollection of unpleasant past events or even depression if they feel that the experimental treatment is not working. From a social point of view participants in the study should not be identified as participants as the illness or condition that they are suffering from may cause stigmatization in their community. The involvement in the study may also cause concern from loved ones if the health issues for the subject were not disclosed to those individuals.

It is the responsibility of the ethics committee to protect participants in clinical trials from unnecessary or avoidable risks by providing their expert assessment of whether the benefits of the clinical investigation out-weigh the risks and potential routes of eliminating or minimizing the risks that are present. The assessment of the clinical trial for ethical implications is another major aspect of the study that the ethics committee will assess. Potential issues such as an overwhelming presence of a power relationship between the study subject and the researcher, inadequate measures taken to protect the privacy and confidentiality of participants, cultural norms or practices that may affect the participant and the economic situation of the participant are some aspects that are assessed by the committee [6].

14.3 Protection of Subjects Through Clinical Trial Design

Pre-clinical data form the foundations for human studies. Before proceeding to early human testing, investigators and reviewers must determine whether the pre-clinical scientific foundation is adequate [4]. The design of the clinical trial also has an impact on the protection of the subjects. Recruitment and consent procedures, measures for protecting privacy and confidentiality, monitoring, data storage and transfer as well as appropriate use of patient sub-groups are elements for a clinical trial to ensure the well being of the clinical trial subject. Starting with the recruitment process the potential subjects must be provided with accurate, clear and concise information regarding their participation in the trial. Most often, recruitment material such as posters, bulletins, and radio or television advertisements must be approved by an ethics committee before they can be used for recruitment. In trials where rewards are offered, they must be offered to the prospective participants in a manner to minimize undue influence to participate in the trial. When introducing the clinical trial, the risk and benefits of participating in the research trial must be presented to persons interested in the trial as well as the inclusion and exclusion criteria for participation. The criteria for determining which patients will be included or excluded from the trial must be justified by the research question as there should not be a specific portion of the general population that is unfairly overrepresented or targeted for human trials and research. On the other hand, it is wrong to neglect or discriminate against individuals or segments of the population in order to gain a favorable outcome. All individuals should have an equal opportunity to participate in research. Groups such as children, the elderly, women, prisoners, ethno-cultural

minorities or those with mental health issues should not be discriminated against and should be treated fairly and equitably in the recruitment process. Measures such as having recruitment material available in another language or transportation to and from the trial site for the elderly should be considered and outlined by the researcher in the clinical trial design.

Once a potential participant shows interest in the clinical trial, additional information regarding the clinical trial is presented to the participant regarding the objective and goal of the trial, what is expected from the participant, procedures that they will undergo during the trial, the risks and benefits of participating, the handling of samples taken, handling of data acquired and alternatives to participating. Information is presented in lay language in order for the average person to fully comprehend the clinical trial. Potential subjects must be given sufficient time to ask questions and voice concerns before making an informed decision to participate. The decision of the individual to participate in the trial must be voluntary and free from coercion. A signed consent document must be attained by the researcher from the participant once they are in agreement to participate. In the event that the study participant is not in the right state of mind to give consent, an appointed guardian must also be involved in the consent process. In the event that the participant is under legal age, it is the responsibility of the parent or guardian to give consent for their child to participate in the clinical trial. This method of attaining consent is beneficial to the participant as they are presented with all aspects of the trial and where their decision to participate in a study is based on a thorough understanding of risks and benefits of participation. Therefore, the protection of human rights and the sanctity of informed consent are critical components of clinical research monitored by human subjects' investigation committees [15].

14.4 Handling of Personal Information and Data

Once the trial has begun and participants provide personal information, the researcher must have the duty to treat personal information respectfully and confidentially. Any personal information gathered must be stored securely and should not be accessible to anyone outside of the trial unless permission has been granted. When the information is no longer needed, it should be destroyed. It is good practice to have no identifying information on any samples collected or study document (such as questionnaires). This can be done by assigning a code for each study participant in order for their identity to be protected. The document linking participants to their study code should only be kept if follow-up contact is required and stored securely if required. Consent documents containing participant's personal information should also be kept separate from information gathered during the study. This procedure is also appropriate for the documentation of any non-written consent process (e.g. field notes). The institution, where the trial is being held, should have

policies in place to determine who has access to personal information about participants throughout the clinical trial. This may include conditions for audits or monitoring of the clinical trial.

All data collected throughout the clinical trial must also be kept under lock and key. Data stored electronically or online must be encrypted and password protected. If data collected will be used in publications, the participant must be informed of this during the consent process. Participant identifiers must never be published unless the participant has specifically consented to the publication. In the event there is a breech in the storage of data or confidential information, the reputation and respectability of the institution as well as clinical research as a whole will be negatively affected.

14.5 The Monitoring Process and Subject Protection During Clinical Trial

Throughout the clinical trial, the rights and well-being of human subjects are also protected through the monitoring process. Clinical trials are monitored for accuracy of the data collected and to ensure GCP guidelines and applicable regulatory requirements are followed. A monitor is selected by the sponsor of the clinical trial to ensure that the study is conducted in compliance with the currently approved protocol or amendments. The monitor will have knowledge of the clinical trial protocol and should have scientific and/or clinical knowledge needed to monitor the trial adequately. Monitoring occurs from the start of the study to evaluate the clinical site, personnel involved in the trial and study documents, throughout the trial, as well as at the time of closure. The monitoring process is a protective factor in the well being of the human participants as they ensure that all safety measures outlined in the protocol are followed.

14.6 Clinical Trial Registration

Clinical trials must be registered in a publicly accessible database before the first subjects are recruited such as the US-based ClinicalTrials.gov and the UK-based International Standard Randomized Controlled Trial Number Register. The general public around the globe has become distrusting of clinical research [16], particularly with some recent high profile cases of scientific misconduct, which has led to the perception that professional integrity on the part investigators and clinicians has deteriorated [17]. Such public misperceptions can impact on clinical trial enrollment and thus hinder innovation. Therefore, restoration of the public trust as well as transparency can be obtained through clinical trial registration. Accordingly, clinical trial registration promotes patient protection and benefit, advances the trust of everyone and is required [17].

14.7 Ending Trials Early

Consideration for terminating a trial prematurely is also related to patient protection. If participants experience adverse effects or if there is clear evidence that the risk to the patient outweigh the benefits, the institutional review boards and monitors can recommend that the trial be stopped early.

14.8 Concluding Remarks

The Declaration of Helsinki offers the best and most effective protection of clinical trial participants, especially in developing countries. The primary role of ethics committees is to uphold ethical principles in order to ensure a high level of protection of individuals that consent for participation in a clinical trial. Approval of a clinical trial is the responsibility of the ethics committees, and is based on the trial design and the way it is reported as well as criteria laid out for the protection of subjects taking part in the clinical trial. An important requirement for the protection of clinical trials subjects in developing countries is transparency on the trial existence, design, protocol as well as the data. The lack of information on clinical trials prospectively, as well as those in progress or even completed in developing countries is presently a major obstacle. A solution to this issue may be to implement a database accessible by the public domain that will ensure that the sponsor and the investigator conduct clinical trials in accordance to ethical principles. Governments and industry leaders are making considerable investments in transforming basic science discoveries into clinical applicability, so-called bench-to-bedside. This will increase the demand for human participation in clinical trials. Accordingly, by ensuring patient protection, the integrity and quality of the clinical trial, and that clinical trials are safe and more transparent; this increased demand will be fulfilled. This will benefit medical science and innovation.

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Chapter 15
Adaptive and Mal-Adaptive Signaling in Cells of the Cardiovascular System: Effect of Obesity-Associated Peptides on Human Blood Platelet Activation

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Abstract Non-communicable, chronic diseases are responsible for ~60 % of all deaths in developing and developed countries. Currently, these diseases account for ~75 % of health care spending in Canada. Although a majority of Canadians express the opinion that health care systems should emphasize prevention strategies, and state supporting funding of prevention programs, the reality is that participation rates in prevention programs are low. Indeed, in North America, 1 in 3 adults are obese. Most disturbingly, 1 in 5 girls and boys between the ages of 6 and 19 is obese and has two or more risk factors for heart disease, including high blood pressure, high cholesterol, diabetes, current smoking and physical inactivity. Research has unequivocally linked obesity, the metabolic syndrome, and other components of "modern life", such as physical inactivity, as factors that increase the burden of chronic disease. The risk of cardiovascular morbidity is significantly augmented by obesity. A number of peptides, including orexins, obestatin, and neuropeptide Y (NPY), play a pivotal role in the regulation of energy expenditure and also affect other systems and cells. Our studies have elucidated some of the mechanisms through which the endothelium and blood platelets integrate these myriad physiopathological stimuli and take advantage of the findings to highlight novel potential therapies to promote adaptive endothelial functions and to reduce the chronic disease-associated mal-adaptive actions of the endothelium.

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15.1 Introduction

Obesity is an unquestionable risk factor for cardiovascular disease (CVD). While several underlying mechanisms linking obesity to CVD have been described, there is no single unifying concept to fully explain this linkage. Increased adipose mass, especially abdominal adiposity, has been shown to represent a strong predictor of CVD and this factor has been proposed to relate to the impact of obesity-related peptide hormones on cells of the cardiovascular system. Of potential importance, abdominal adipocytes have been reported to synthesize and deliver to the blood stream vasoactive peptide hormones, including leptin and adiponectin, and evidence has accumulated correlating differences in levels of these peptides and CVD. Recently we reported that some of these, leptin [1], adiponectin or ghrelin [2] could influence platelet and vascular endothelial cell functions. Other peptide hormones that control appetite and metabolism are also known, including orexins, obestatin, and neuropeptide Y (NPY) [3]. Herein we report on the potential that these important peptides also have on platelet activity to test for their possible involvement in linking obesity to CVD.

Orexins (A and B), formerly known as hypocretins 1 and 2, are hypothalamic neuropeptides that act through actions on two homologous G protein-coupled receptors (GPCRs); orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R) [4–6]. Orexin A, the more potent of the orexins, is excitatory in the central nervous system, stimulating sympathetic activity, arousal, spontaneous physical activity, thermogenesis and food intake [6, 7]. Encoded by the *ghrelin* gene, the 23 amino acid peptide; obestatin; suppresses appetite by opposing the effects of ghrelin on food intake; a dual system of appetite control that may explain why ghrelin-null mice have normal appetites. Obestatin is primarily secreted by the gastrointestinal mucosa, and acts within the gut as well as at the hypothalamus and the pituitary [8], Initially thought to act by activating GPR39, an orphan GPCRGPR39 [8], further studies will be required to identify this peptide hormone's actual receptor protein [9]. Neuropeptide Y is an orexogenic peptide secreted by the hypothalamus which has both central and peripheral effects. Six NPY receptors, designated Y1 through Y6, are known [10, 11]. Neuropeptide Y is present in platelets and has been associated with increased angiogenesis and formation of atherosclerotic-like lesions [10, 12].

15.2 Methods

15.2.1 Preparation of Platelet Rich Plasma and Aggregation Studies

Platelet aggregations were determined as previously described [1]. Briefly, blood was obtained from healthy volunteers who were drug-free for a minimum of one week. Blood was anti-coagulated with heparin (15 U/ml) and platelet rich plasma

(PRP) was obtained by centrifugation (284 g, 15 min at room temperature). Platelet poor plasma (PPP) was obtained by PRP centrifugation (2,750 g, 5 min at room temperature). Aliquots of PRP (500 µl, 2×10^8 platelets/ml) were incubated in siliconized glass disposable cuvettes with peptide hormones (orexin A, obestatin, NPY (Phoenix Pharmaceuticals, Belmont, CA), or vehicle for 5 min at 37 °C and then aggregated (3 min) by addition of ADP (2 µM) in a dual chamber optical aggregometer (490-2D, CHRONO-LOG Corporation, Haverton, PA).

15.2.2 Statistical Analysis

Data are presented as means ± S.E.M. from a minimum of three independent experiments within which individual platelet samples were tested in triplicate or quadruplicate. Statistical differences between conditions were determined using the Student's t-test with $P < 0.05$ considered significant.

15.3 Results

15.3.1 Effect of Orexin A on Platelet Aggregation

Orexin A (100–500 ng/ml) significantly inhibited ADP-induced aggregation of human platelets. Thus, while ADP (2 µM) aggregated platelets by 62 ± 2 %, in the presence of the lower concentration of orexin A tested (100 ng/ml), this effect of ADP was reduced such that platelet aggregation was 55 ± 2 % ($n > 3$ experiments; $P < 0.05$). The higher dose of orexin A tested did not further inhibit ADP-induced platelet aggregation and in fact was equivalent at inhibiting platelet aggregation.

15.3.2 Effect of Obestatin and NPY on Platelet Aggregation

In contrast to the ability of orexin A to inhibit ADP-induced aggregation of human platelets, neither obestatin (100–1,000 ng/ml) nor NPY (100–1,000 ng/ml) impacted ADP-induced platelet aggregation. Indeed, while ADP (2 µM) aggregated human platelets by 64 ± 2 %, values in the presence of obestatin (100, 500 or 1,000 ng/ml) were 62 ± 2 %, 64 ± 2 % or 65 ± 2 %, respectively ($n > 3$; $P > 0.05$). Similarly, compared to aggregation induced by ADP, aggregation of human platelets in the presence of NPY (100, 500 or 1,000 ng/ml) were unaffected (~2 ± 2 % at all doses tested, $n > 3$ experiments; $P > 0.05$).

15.4 Discussion

Several mechanisms have been proposed to link obesity and CVD. Among these, an interaction between the endocrine and cardiovascular systems is popular. In this context, a significant number of peptide hormones are known to influence satiety and energy expenditure. In addition, these hormones also impact functions of cells of the cardiovascular system. In two previous reports, we described the effect of three hormones; leptin, adiponectin, and ghrelin on platelet function [1, 2]. In these earlier studies we reported that leptin, but not adiponectin or ghrelin potentiated ADP-induced platelet aggregation. Indeed, we identified an effect of leptin on cAMP hydrolysis as a likely basis for its effects. Herein, we report that while orexin A could potentiate ADP-induced aggregation of human platelets that obestatin and NPY did not. Since orexin A was known to stimulate the sympathetic nervous system, we had predicted that this peptide hormone would enhance platelet activation. In marked contrast, our data indicated that orexin A inhibited ADP-induced platelet aggregation. Although of potential physiological or pathological importance, the molecular basis for this discordant observation will require further work. Similar to our report that ghrelin did not influence platelet function [2], obestatin also was without effects in our studies. Despite the fact that NPY is known to be expressed in platelets, and that it can compromise certain functions of cells of the cardiovascular system in experiments [11, 12], our studies showed that NPY did not augment platelet activity. This observation is consistent with the likely conclusion that NPY does not act as an autocrine factor for platelets, but rather is released from these cells to act on other systems of the cardiovascular system. Taken together, our data indicate that only orexin A might have a protective effect against cardiovascular morbidity through its inhibitory effect on platelet activity. Further studies are recommended to explore the mechanism through which orexin A shows this inhibitory effect.

15.5 Conclusion

In combination with our previous work in which we report the effects of leptin on platelets and human VECs, we believe that these findings further reinforce the idea that obesity-related peptides directly impact functions of cells of the cardiovascular system. Indeed, we conclude that a direct link is highly likely between the effects of these peptide hormones on control of satiety, hunger, weight gain and cardiovascular disease-associated increases in morbidity and mortality.

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Chapter 16
Spontaneous and Induced Mutagenesis: The Necessity and Possibilities of Its Prevention with the Grape Polyphenolic Concentrate Enoant

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Abstract The use of antioxidants from natural products for gene reparation may be better than those of a synthetic origin due to their low toxicity and apparent absence of adverse side effects. Grape polyphenols contain antioxidants having antimutagenic qualities. Particularly, the food concentrate Enoant, rich in grape polyphenols and extracted from Cabernet Sauvignon grapes, is a valuable source of natural antioxidants. In the current study, we investigated the possibility of using Enoant to modify the cytogenetic changes caused by spontaneous (due to aging) or induced (by thyroid hormone T4 and by ionizing radiation) mutagenesis. The cytogenetic effects of Enoant were characterized depending on dosage, application time, age and sex.

16.1 Introduction

Mutations are quantitative or qualitative genome changes, which are passed from cell to cell and from generation to generation by genome replication. The mutations are classified by the level of genetic damage (e.g., genome, chromosomal, and gene mutations) and by cell types (e.g., mutations can occur in reproductive and somatic cells) [1, 2]. Reproductive cell mutations result in a mutant organism and this can be passed on to the next generation by reproduction. Such mutations can lead to the birth of children with congenital diseases. As a rule, reproductive cell mutations do not gravely influence their carrier organism nor do they increase the burden of mutation. Mutations in fetal somatic cells influence the development of various organs and systems. This depends upon the appearance of the mutation at different

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ontogenic stages. It is known that somatic cell mutations lead to tumors. The additional influence of exogenous factors may increase the mutation process and the accumulation of pathologic mutations. It may also change the expression of the genetic structure by the new ecological conditions [3–5]. The burden of genetic structural damage manifests itself in a reduction in the capacity of the mutation carrier to adapt to life conditions, namely, in the capacity of the living body to leave viable offspring in a certain environment. The medical consequences of mutations are also very important. Mutations lead to a decrease in the viability of the organism because somatic cell changes underlie the aging process [6–9]. There is also a strong connection between mutagenesis and oncogenesis. There are important cytogenetic defects resulting in malignant cell transformation and chromosomal abnormalities, which cause tumor progression and metastasis [10]. Induction of gene and chromosomal mutations can lead to proto-oncogene activation and/or gene suppressor loss [11].

Reproductive cell damage can also lead to early termination of pregnancy. If a miscarriage occurs early in the term, the woman may be unaware of this. This may, in turn, lead to an increase in the quantity of infertility in couples; such a tendency is being observed world-wide, including Ukraine [12]. The birth of children with congenital malformations is a very important problem. It is considered that 50 % of congenital pathology is caused by injuries during pregnancy [13].

As a rule, mutations are balanced by the repair of gene structures and/or the elimination of cells with damaged genetic material. Because of this, stability is retained. Mutagenic factors may create new mutations in such a quantity that the reparation systems are not able to eliminate all the defects. In this case, mutations may induce pathological results.

There are various physical, chemical and biological agents, which can change the function and morphology of hereditary structures (ionizing and non-ionizing radiation, pesticides, products of smoking, viruses, etc.) [14–17]. External impacts lead to the toxic effects on genes, which can cause oxidative stress. Activated oxygen species can appear in cells and exceed the capacity of the antioxidant system. A shortage of antioxidants can result in membrane damage (including the membrane of genetic structures) by activated oxygen metabolites [18, 19].

The negative effects of induced mutagenesis require application of each and every means of prophylaxis. The most effective way is to prevent the release of mutagens into the environment and to maintain safety hazard levels and concentrations. This is attained through the observance of state sanitary and hygiene standards. However, there is a mutagenic influence of certain factors like non-ionizing radiation and some medications that cannot be prevented in this way [17]. In addition, a major part of the population suffers from chronic stress, which has been shown to cause hormone imbalance and chromosome damage [20–23].

If the mutagenic load is increased, endogenous protective mechanisms cannot always completely neutralize these mutations and prevent their negative influence. However, we have recently become aware of the importance of saturating an organism with antioxidants as a mechanism to prevent and/or treat spontaneous and induced mutagenesis. There is an opportunity to neutralize the late consequences of some mutagenic environmental factors by the anti-mutagenic qualities of some

substances (including food components). Today, it may be possible to negate the influence of induced mutagenesis through not only the regulation of the concentration of toxic genetic substances and their levels in the environment, but also by the consumption of food with certain level of natural antioxidants [9, 24–26]. Natural substances are better for gene reparation because of their low toxicity [26, 27].

The dietary concentrate of grape polyphenols Enoant, made from Cabernet Sauvignon grapes, contain antioxidants that have antimutagenic qualities. The application of antimutagenic substances to people needs to be studied for safety and efficacy. Therefore, the aim of this study was to investigate the possibility that Enoant can modify the cytogenetic changes caused by spontaneous (aging) and induced (thyroid hormone – T4 and ionizing radiation) mutagenesis. It was also important to characterize the anti-cytogenetic effects of Enoant as a function of dosage, application time, and age and sex of the animals.

16.2 Materials and Methods

The main part of investigation was carried out on white three-month old nonlinear male rats, which were housed in a usual (non-standardized) vivarium regimen. Vivarium life conditions, e.g. light regimen, microclimate, etc., and the residence time of control and test animals were the same throughout the study. To study the influence of age, 5-months rats were also studied.

The liver was an object of study. Liver mitotic index is 0.01–0.02 % [28] meaning that the adult rat liver cells division is 100 times slower than marrow cells division. Thus, the elimination of hepatocytes with damaged chromosomes is slow. At the same time, the liver is able to regenerate actively after a hepatectomy. This enables observation of the cell division and the status of chromosomes. Liver cells are synchronized and primarily stay in G_0 cell cycle phase [29]. The liver is a convenient object for mutagenesis study because it has high spontaneous levels of hepatocytes with chromosome aberrations, and these aberrations accumulate in aged animals comparing to the bone marrow [30, 31].

Enoant was made according to specifications TU 00334830.018-99 (Ukraine) from peals and seeds of Crimean Cabernet Sauvignon grapes. The concentrate contains grape polyphenols and microelements. The choice of dosage was based on the previous clinical studies of prophylaxis and treatment of bronchopulmonary diseases by Enoant [32].

Spontaneous mutagenesis was studied after oral introduction to 3-months animals at daily dosages of 0.12, 0.25, 0.52, 0.78, and 1.04 ml/kg of body weight during 30 days. In 5-months animals, the daily dosage was 0.52 ml/kg during 30 days. The concentrate was dissolved in boiled water. The daily dosage consisted of two parts.

The anti-cytogenetic effects of Enoant were studied under the conditions of T_4 induced mutagenesis. T_4 (“Reanal”) was dissolved in 0.1 M KOH solution and was administered intramuscularly once a day. Its dosage was 10 μg per 100 g of body weight during 14 days.

The daily dose of 0.78 ml/kg of Enoant has been introduced during 14 days simultaneously with T_4 or during the next 14 days after T_4 introduction. The duration of the treatment was chosen according to the accumulation in rat hepatocytes with chromosome aberrations [30, 31] and to the ability of T_4 to increase the frequency of cells with aberrations [22]. Control animals received 10 ml/kg T_4 per 100 g of body weight intramuscularly once a day during 14 days.

Total single ionizing irradiation (dosage 250 cGy) was also used as a mutagen because pathologic anaphase and metaphase may be indicators of the effects of the radiation (at dosages less than 3 Gy) [31]. The daily dose of 0.78 ml/kg of Enoant was introduced during 30 days before irradiation or after irradiation from the 1st to the 30th day or from the 4th to the 30th day.

Control animals received boiled water. The water volume and administration protocol were the same as those used for the Enoant group. The frequency of hepatocytes with chromosome aberrations was investigated among rats during first day, which did not receive T_4 and Enoant (vivarium control).

Two-thirds of the rat liver were ablated and fixed with formalin. Calculations of the mitotic index (MI) were made. Animals were sacrificed by removal of the cervical vertebrae 30 h after the operation, i.e. in the period of maximal mitosis of hepatocytes. The caudal segment of the liver was fixed by formalin [33, 34]. Histological microscopic sections were colored by Felgen's method [35]. First division hepatocyte cells with chromosome aberrations (fragments, bridges) were counted in a "blinded" manner. This was performed for 100 hepatocytes during late anaphase and early telophase. The mitotic index (MI) was estimated for cells in pro-, meta-, ana- and telophase per 1,000 hepatocytes.

16.3 Result and Discussion

16.3.1 Spontaneous Mutagenesis

The level of hepatocytes with chromosomal damage at the beginning of the experiment corresponded to their calendar age. If the concentrate was administered at a dose of 0.52 mg/kg daily during 15 days, the frequency of hepatocytes with chromosome aberrations was the same as in the control group (Table 16.1).

During 30 days, the spontaneous level of hepatocytes with chromosomal aberrations increased (Table 16.2). Enoant administered at dosages of 0.52, 0.78, and 1.04 mg/kg during 30 days decreased the frequency of cells with chromosome aberrations. Daily dosages of 0.12 and 0.25 mg/kg were not effective.

The daily administration of concentrate did not result in an improved cytogenetic effect. Therefore, Enoant may reduce the biological age of the rats by reducing the frequency of hepatocytes with chromosome aberrations by spontaneous

Table 16.1 The frequency of hepatocytes with chromosome aberrations as a function of Enoant administration to three-month male rats (0.52 mg/kg during 15 days)

Group	Enoant dosage, mg/kg	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)	P value
Control	–	9	900	8.0 ± 0.6	–
Test	0.52	9	900	8.0 ± 0.4	> 0.1

Table 16.2 Frequency of hepatocytes with chromosome aberrations under different dosages of Enoant administered to 3-month male rats during 30 days

Group	Enoant dosage, mg/kg	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)	P value
1. Vivarium control	–	10	1,000	7.0 ± 0.6	–
2. Control	–	10	1,000	9.6 ± 0.4	1 vs. 2 <0.01
3. Test	0.12	9	900	8.6 ± 0.5	3 vs. 2 >0.1
4. Test	0.25	10	1,000	8.4 ± 0.6	4 vs. 2 >0.1
5. Test	0.52	10	1,000	5.3 ± 0.2	5 vs. 2 <0.001
6. Test	0.78	7	700	4.2 ± 0.7	6 vs 2 <0.01
7. Test	1.04	7	700	3.9 ± 0.9	7 vs 2 <0.01

Table 16.3 Enoant influence on frequency of cells with chromosome aberrations when administered to male rats at a daily dosage of 0.52 ml/kg for 30 days (when investigated at different times after hepatectomy)

Fixation time after hepatectomy (h)	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)
29	10	1,000	5.8 ± 0.5
30	10	1,000	5.8 ± 0.5
31	10	1,000	6.0 ± 0.8

mutagenesis. The frequency of hepatocytes with chromosome aberrations was lower with Enoant administration than before the experiment, which supports an adaptative mechanism [36]. This anti-mutagenic effect depends not only on the concentrate dosage, but on the time of administration (Tables 16.1 and 16.2). It can be concluded that the concentrate may be used to slow the ageing processes.

It is known that reduction of the frequency of cells with chromosomal aberrations induced by spontaneous mutagenesis may occur as a result of changes in aberrative cell cycle times. It was shown that Enoant did not change the speed of the aberrative cell cycle (Table 16.3).

Table 16.4 Mitotic index by administration of Enoant at different dosages to 3-month male rats during spontaneous mutagenesis

Group	Administering time, days	Enoant, ml/kg	Mitotic index per 1000 cells (mean ± s.e.m.)	P value
1. Control	–	–	0.55 ± 0.05	–
2. Test	30	0.78	0.40 ± 0.05	1 vs. 2 >0.01
3. Test	30	1.04	0.43 ± 0.02	1 vs. 3 >0.1

Table 16.5 The frequency of hepatocytes with chromosomal aberrations after administering 0.52 ml/kg Enoant to 5-month male and female rats for 30 days

Groups	Sex	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)	P value
Control	Male	8	800	13.1 ± 1.2	–
Test	Male	4	400	6.7 ± 1.0	0.001
Control	Female	8	800	10.0 ± 0.9	–
Test	Female	7	700	6.2 ± 0.3	> 0.01

Enoant did not change hepatocytes MI (i.e. the number of divided cells) before hepatectomy (Table 16.4). This suggests that there was no stimulation of the elimination of hepatocytes with chromosome aberrations.

The Enoant effect is shown at first mitosis after partial hepatectomy and also in tissue which does not regenerate. This would be expected to decrease the probability of elimination. It is possible that Enoant prevents chromosomal damage in hepatocytes or improves their reparation.

It is known that the reparation system in genetic structures works poorly in aged organisms [37] and there are also sex differences in the reparation systems. Accordingly, the anti-cytogenetic effects of Enoant were studied in male and female animals (Table 16.5).

After administration of 0.52 ml/kg of Enoant to five-month male and female rats for 30 days, the frequency of hepatocytes with chromosomal aberrations decreased in all groups. This suggests that age and sex do not influence the anti-cytogenetic effect of Enoant.

16.3.2 Enoant Cytogenetic Effect by Thyroxin-Induced Mutagenesis

Simultaneous administration of Enoant and T4 reduces the frequency of hepatocytes with chromosome aberrations by 4.4 times in comparison to the control group (administering only T_4) (Table 16.6). The frequency of hepatocytes with chromosomal aberrations was also 1.3 times lower than its initial level (before the beginning of the test) (Table 16.6).

Table 16.6 The frequency of hepatocytes with chromosomal aberrations after simultaneous administration of 0.78 ml/kg of Enoant and 10 mkg per 100 g of body weight of T_4 to 3-month male rats for 30 days

Groups	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)	P value
T_4	8	800	18.4 ± 1.3	–
Enoant + T_4	6	600	4.2 ± 0.4	<0.001

Table 16.7 The frequency of hepatocytes with chromosomal aberrations after administering 0.78 ml/kg of Enoant for 14 days after administering 10 mkg per 100 mass grams of T_4 for 14 days to 3-month male rats during 30 days

Groups	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)	P value
T4 control	8	800	18.4 ± 1.3	–
T4 + Enoant	8	800	4.0 ± 0.6	<0.001

Administration of Enoant for 14 days after administering T_4 for 14 days resulted in the frequency of hepatocytes with chromosomal aberrations being reduced by 4.5 times in comparison with the control levels (Table 16.7).

A surplus of thyroid hormones can cause cytogenetic damage [22], whereas Enoant application may minimize it. Therefore, it could be useful to include Enoant in a treatment scheme of hyperthyroid.

16.3.3 Enoant Cytogenetic Effect by Mutagenesis Induced by Total Single Ionizing Irradiation (Dosage 250 cGy)

There were 30.2 ± 1.4 % cells with chromosomal aberrations on the 30th day after irradiation (dosage 250 cGy) (Table 16.8). Enoant administration for 30 days after irradiation decreased the frequency of hepatocytes with chromosomal aberrations by 1.6 times in comparison with the control group (the control group was irradiated, but did not receive Enoant). The decrease in the frequency of cells with chromosomal aberrations was also corroborated after testing the concentrate by administration from the 4th to the 30th day after irradiation (the decrease in frequency was more evident).

It is known that during the first day after irradiation of rats, the endogenous antioxidants in the tissues are becoming mobilized to prevent the deleterious effects of irradiation [38]. The effects of Enoant administration from the 4th to the 30th day after irradiation confirm this hypothesis. However, the level of reparation may be limited by the receptor number and the saturation level. Under such conditions, the effects of exogenous antioxidant may be less pronounced. The administration of Enoant for 30 days before irradiation did not change the frequency of hepatocytes with chromosome aberrations. This may explain why it is possible that

Table 16.8 The frequency of hepatocytes with chromosomal aberrations after administration of Enoant to 3-month male rats after a single ionizing irradiation (dosage 250 cGy)

Group	Influence time, days	Enoant dosage, ml/kg	Number of rats	Number of cells in ana- and telo-phase	Cells with aberrations, % (mean ± s.e.m.)	P value
1. Control	30	–	10	1,000	9.0 ± 1.2	–
2. 250 cGy	30 (water after irradiation)	–	10	1,000	30.2 ± 1.4	1 vs. 2 <0.001
3. 250 cGy	30 (water before irradiation)	–	9	900	27.0 ± 2.5	1 vs. 3 <0.001
4. 250 cGy	1-30 (after irradiation)	0.78	10	1,000	18.9 ± 1.3	2 vs. 4 <0.001
5. 250 cGy	4–30 (after irradiation)	0.78	10	1,000	10.2 ± 1.4	2 vs. 5 4 vs. 5 <0.001
6. 250 cGy	1–30 (before irradiation)	0.78	10	1,000	24.1 ± 1.9	3 vs. 6 >0.05

the mechanism for reducing damaged cells is connected with the neutralization of free radicals. This is also confirmed by investigations of the antioxidant characteristics of the grape polyphenol enomelanin [39].

According to the presented results, the concentrate has medicinal, non-protective characteristics concerning the irradiation influence. It is possible to apply Enoant as a support for tumor irradiation. The different molecular mechanisms responsible for the effects are mentioned above. It is clear that the use of Enoant is necessary and desirable for the prophylaxis of spontaneous and induced mutagenesis.

16.4 Conclusion

- Enoant may reduce the biological age of rats due to reducing the frequency of hepatocytes with chromosome aberrations by spontaneous mutagenesis. The concentrate may be used for slowing the ageing process.
- The cytogenetic effects of Enoant may minimize thyroxin-induced mutagenesis; so, it is useful to include Enoant in the treatment of thyroid diseases, which also may decrease tumor risk.
- The medicinal characteristics of Enoant shown here are related to the irradiation influence (according to the frequency of cells with chromosomal aberrations). It may be possible to apply Enoant as a support for tumor treatment.
- There was no protective influence of Enoant during radioactive damage (according to the frequency of cells with chromosomal aberrations).

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Chapter 17
Searching for New Antimicrobial Targets: Na^+ Cycle in Energetics of Bacterial Pathogens

Pavel Dibrov

Abstract Outbreaks of microbial infections (tuberculosis, cholera, etc.) endangering lives of civilians and military personnel alike are inevitable consequences of social and military crises. The situation is further exacerbated by the current global crisis of antimicrobial therapy caused by common misuse of broad-range antibiotics and the resulting proliferation of drug-resistant strains. Despite the resurrected interest in alternative approaches, such as the development of adjunctive immunotherapy, search for new targets for prospective antimicrobials apparently remains the most viable option in the management of microbial infections. A consensus is emerging that a new generation of antimicrobial remedies should include precisely targeted, ideally – pathogen-specific drugs. As it is evident from cross-genome comparisons, Na^+ pumping systems of different types (comprising a "sodium cycle" in bacterial membrane energetics) are "overrepresented" in microbial pathogens. This might be due to a peculiar evolutionary relationship between the "mainstream" type of bacterial energetics based on the transmembrane circulation of proton (H^+ cycle) and more archaic Na^+ cycle. Wide spreading of elements of Na^+ cycle among different pathogens makes these systems attractive targets for prospective development of novel, narrowly targeted antimicrobials. In this communication, a "target potential" of the primary respiratory Na^+ pump, NQR, and secondary Na^+ pumps, NhaA/B, is discussed.

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17.1 Current Crisis of Antimicrobial Therapy

It seems, as the broad consensus has been reached among both health care practitioners and academics, that the era of antibiotics might be at its end [1]. The present crisis was precipitated by decades of global misuse of broad-spectrum antibiotics and resulting proliferation of multi-drug resistant strains. Adjunctive immunotherapy and other alternative approaches have serious limitations [2] and are still much less effective compared to administration of antimicrobials. Thus search for new prospective antimicrobial targets remains one of major available options. High-throughput screening protocols in conjunction with extensive chemical libraries and structure-assisted drug design are aimed at development of new generation of narrowly targeted, preferably – pathogen-specific remedies that will avoid pitfalls of the currently employed antibiotic-based management of microbial infections [3]. In this context, membrane proteins forming the core of Na^+-dependent energetics in bacteria are particularly interesting as prospective drug targets.

17.2 Na^+ Cycle in Energetics of Bacterial Pathogens

17.2.1 Na^+ Cycle vs. H^+ Cycle

In vast majority of free-living bacteria, as well as in mitochondria and chloroplasts, the transmembrane electrochemical gradient of H^+ (proton-motive force, PMF) generated across the cytoplasmic membrane by primary H^+ pumps may be directly used for ATP synthesis, solute accumulation, ion exchange, motility, reverse electron transport, etc. [4]. Some bacteria, however, possess in their membranes primary Na^+ pumps generating the transmembrane electrochemical gradient of Na^+ (sodium-motive force, SMF). In such species as *Propionigenium modestum*, *Malonomonas rubra*, and *Caloramator fervidus* SMF completely substitutes for PMF as a primary energetic source [5–7]. Sodium ion circulation (Na^+ cycle) have been initially rationalized as an adaptation to life at high external pH or high temperatures, where maintaining the high levels of PMF is difficult [4, 8, 9]. However, not all alkaliphiles and hyperthermophiles rely on Na^+ cycle for energy transduction; on the other hand, quite a number of mesophilic species possess at least one primary Na^+ pump. Of note, secondary ion pumps, Na^+/H^+ antiporters, which are interconverting PMF and SMF, are universal components of bacterial membranes irrespectively of the nature of primary coupling ion (Fig. 17.1).

In addition to their immediate role in energy conversion, Na^+/H^+ antiporters are essential components of the Na^+ and H^+ homeostasis in *Escherichia coli* [10] and many other bacterial and eukaryotic cells [11]. In addition, under the energetic stress (when primary H^+ pumps are inactive), Na^+/H^+ antiporters, operating in the reverse mode, would expel internal protons in exchange for extracellular Na^+ ions, thus using pre-existing SMF to prevent the PMF from an immediate dissipation.

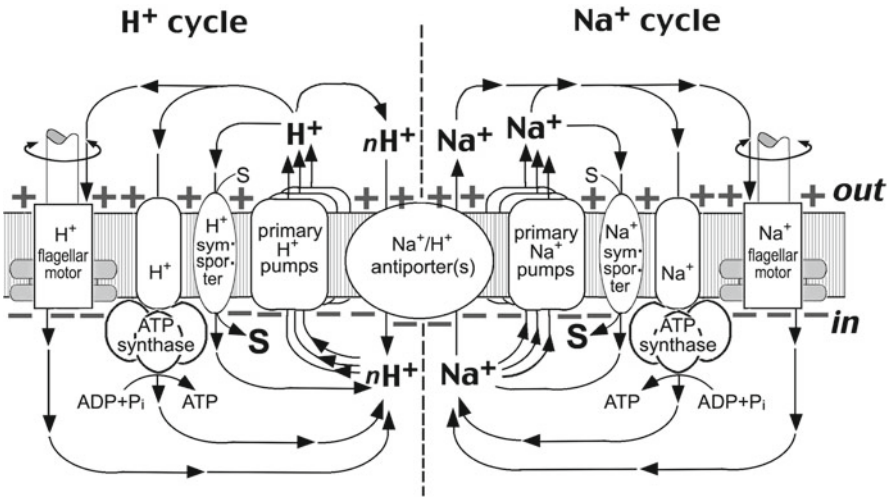

Fig. 17.1 Sodium (*right side*) and proton (*left side*) cycles in membrane energetics of bacteria. Na^+/H^+ antiporters connect both cycles by interconverting PMF and SMF. Operation of primary pumps swiftly charges the membrane (cytoplasm negative) due to the low electric capacitance of the lipid bilayer Electrogenic Na^+/H^+ antiporters (exchanging more than one Na^+ ion per each H^+, $n > 1$) can use the resulting difference of electric potentials ($\Delta\psi$) in addition to the osmotic component of the PMF, ΔpH, to create significant transmembrane gradient of Na^+. In alkaline environments, this mechanism, using the $\Delta\psi$ generated by primary Na^+ pumps, efficiently acidifies the cytoplasm, ensuring the survival of bacteria. Certain elements of Na^+ and H^+ cycles may co-exist in the same membrane

This function of Na^+/H^+ antiport (as an energetic buffer for the PMF) has been demonstrated, for example, in halophylic *Halobacterium salinarium* (see [8] and references therein).

17.2.2 Na+ Cycle in Evolution

Recent work, combining metagenomic analysis with systematic comparison of the available structural data, presented several lines of evidence that the H^+ cycle is a relatively late evolutionary acquisition while the Na^+-based membrane energetics is its evolutionary predecessor [12]. The driving force behind the transition from primary Na^+ to primary H^+ pumping was an enormous thermodynamic gain of assembling the primary redox H^+ pumps into electron-transport chains covering the redox span of ~1.2 eV from organic substrates to such high-potential electron acceptors as oxygen, nitrate, sulfate, or sulfite [12]. According to this logic, the Na^+-cycle remained preserved only in ecological niches where the high-potential electron acceptors are not available and the thermodynamic advantage of H^+-coupled electron transfer could not be realized. In particular, such niches are occupied by

anaerobic hyperthermophiles and alkaliphiles that derive energy exclusively from fermentations of different kinds. Since biological membranes are much more permeable for protons than for sodium ions, especially at high temperatures due to the elevated H^+ leakage [12], it is hard to maintain sufficient levels of PMF under these conditions. In alkaline media, transmembrane ΔpH and $\Delta\psi$ have opposite polarities thus once again resulting in diminished levels of the PMF. This is why here, in the absence of efficient electron transport, the Na^+-based energetics is prevailing over its H^+-based type.

17.2.3 Na^+ Cycle Is "Overrepresented" in Pathogens

An earlier cross-genome comparison revealed that primary Na^+ pumping enzymes of different types (comprising the core of the Na^+ cycle in bacterial membrane energetics) are "overrepresented" in microbial pathogens [13]. Noticeably, the complete Na^+ cycle or at least its key components present in the pathogens of very different biology and dissemination/colonization strategies. For example, the causative agent of syphilis, spirochete *Treponema pallidum*, does not have primary H^+ pumps at all; its energetics is exclusively based on the SMF, generated by its Na^+-motive oxaloacetate decarboxylase, rather than on "conventional" PMF [13]. Obligate intracellular parasites, *Chlamydia trachomatis* and *Chlamydia pneumoniae*, also possess a complete sample of the Na^+ cycle, which seems to be playing a crucial role in their pathogenicity [14]. Classic water-born pathogen, *Vibrio cholerae*, uses PMF for the membrane-linked ATP synthesis [15], but its membrane contains not only the aerobic Na^+-translocating NADH:ubiquinone oxidoreductase, NQR, which is the principal respiratory ion pump in this organism [16], but also a Na^+-translocating oxaloacetate decarboxylase (OAD), which couples anaerobic citrate fermentation to Na^+ extrusion [5, 7, 12, 13, 16]. Both enzymes directly energize motility in *V. cholerae* as well as an impressive battery of Na^+/H^+ antiporters and Na^+-substrate symporters (see Fig. 17.1, also [13] and references therein). The tandem NQR-OAD, capable of generating and maintaining the SMF in both aerobic and anaerobic environments, could also be found in such diverse pathogens as *Porphyromonas gingivalis* (causing periodontitis), *Pseudomonas aeruginosa* (lung and skin infection), *Salmonella typhi* (typhoid fever), *Klebsiella pneumonia* (pneumonia), and others [13]. Sole NQR is even more common, being found not only in very many marine bacterial species probed so far [17], but also in a number of human and animal pathogens [12, 13]. In particular, it is present in such lethal pathogen as *Yersinia pestis* (cases of *V. cholerae* and *Y. pestis* will be analyzed in some more detail below).

This widespread distribution of the elements of Na^+ cycle among pathogenic bacteria could be due to several possible reasons. For the energetics of invading pathogenic microorganism, innards of an animal host represent a rather challenging environment, where high-potential electron acceptors (oxygen, nitrate, sulfate,

or sulfite) typically are not available. This precludes thermodynamic advantages of the proton-based electron transport mentioned in the above Sect. 17.2.2. Furthermore, the PMF on the membrane of invading bacterium is targeted by the host defense mechanisms that impair integrity of the bacterial membrane, such as generation superoxide radicals or secretion of high levels of detergent-like compounds (bile salts, etc.). Such energetic stress (leaky membrane in the absence of effective electron transport) would create a selective pressure preserving the archaic Na^+ cycle in the energetics of pathogens. On the other hand, preservation of primary Na^+ pumps, together with extended system of Na^+/H^+ antiport, in pathogens with a free-living phase of the life cycle, may help to meet a different challenge. In the case of *V. cholerae*, inhabiting estuarine ecological niches, this challenge is represented by wild swings of pH (reaching up to 9.5) and salinity occurring in estuaria [18]. Here the Na^+ cycle ensures bacterial survival at high pH, by removal of toxic Na^+ ions from the cytoplasm and coupled acidification of bacterial interior. Since the total level of PMF in alkaline media might be lowered quite significantly due to the necessity of maintaining the cytoplasmic pH reasonably close to neutrality, combination of the $\Delta\psi$-generating primary Na^+ pump(s) with electrogenic Na^+/H^+ antiporter(s) seems to be the only viable solution (Fig. 17.1). Last but not least, the striking similarity between the salt composition of blood and sea water could promote the development of similar adaptation mechanisms in both blood-borne pathogens and marine microorganisms, or, alternatively, acquisition of the corresponding genes through horizontal gene transfer.

17.2.4 Chemiosmotic Targets for Novel Antimicrobials: Precedents

Unfortunately, membrane proteins-generators of the proton-motive and sodium-motive force as potential targets for antimicrobials have generally been overlooked so far, thus precedents here remain precious few. Being phylogenetically restricted by marine species and a number of human and animal pathogens, Na^+-translocating NADH:ubiquinone oxidoreductase, NQR, seems to be an appealing candidate as a prospective "precise" target for antimicrobials. Curiously, the NqrA subunit from *Actinobacillus pleuropneumoniae*, the causative agent of pleuropneumonia in swine, turned out to be immunogenic in infected animals [19]. Despite the fact that NQR resides in the cytoplasmic membrane of bacteria rather than in the outer membrane, the serum of convalescent pigs, infected with *A. pleuropneumoniae*, contained anti-NqrA antibodies. Although it is hard to see how targeting a component of the inner bacterial membrane with antibodies could help in fighting the infection, the above finding points toward NQR subunits as possible vaccine candidates. Interestingly, the NqrA protein from another pathogen, *Porphyromonas gingivalis*, has been patented in Australia as a "50 kD antigen PG1" (patent AU 98/01023, GenBank accession number AF144076).

Another example of primary ion pump as a prospective drug target is provided by the H^+-motive F_0F_1-type ATP synthase of *Mycobacterium tuberculosis*. By adopting a medium-throughput screening, it has been discovered that a certain diarylquinoline derivative, R207910, targets exclusively the *c* subunit (product of the *atpE* gene) of mycobacterial enzyme without any effect on human ATP synthase [20]. R207910 showed very high bactericidal activity against mycobacteria (including *M. ulcerans* causing Buruli ulcer, the emerging human infection for which surgery remains the only available cure) but not other microorganisms; most important, the drug was active against multi-drug resistant strains of *M. tuberculosis* [20]. In the context of the present communication, the importance of this finding is two-fold: (i) it shows that arresting the membrane-bound ATP synthase may be fatal for bacterial pathogens despite of the remaining possibility of substrate-level phosphorylations; and (ii) since the *c* subunits of F_0F_1-type ATPases determine its ion specificity (i.e., H^+ *vs*. Na^+), one may expect that efficient and highly specific inhibitors could be found/developed for Na^+-motive ATPases of F_0F_1-type as well as structurally similar A/V-type that are encoded in genomes of such pathogens as *Chlamydiae*, *T. pallidum*, *Streptococcus pyogenes*, or the ethiological agent of Lyme disease, *Borrelia burgdorferi* [15].

17.3 Cases of *Vibrio cholerae* and *Yersinia pestis*

17.3.1 Different Life Strategies of V. cholerae and Y. pestis

Yersinia pestis is an invasive, facultative intracellular pathogen that causes bubonic plague, undoubtedly the most devastating bacterial infection known, which claimed more than 200 million of victims over the course of written history. The extraordinarily high lethality of plaque apparently reflects both a relatively short evolutionary history of *Y. pestis* (which diverged from the enteropathogenic *Yersinia pseudotuberculosis* within the last 20,000 years [21]) and the most unusual dissemination strategy adopted by this relentless pathogen. Due to the massive loss of functional genes and consecutive metabolic simplifications [22], *Y. pestis* presently has a tightly closed 'flea-mammalian host-flea' cycle, in which death of the already infected host as a prerequisite for a further spread of yersinia-carrying fleas [23]. Therefore bacterial dissemination only occurs in highly homeostatic environments of animal body fluids and tissues. This biochemical feature apparently makes *Y. pestis* highly sensitive to the ionic composition of its microenvironment.

In sharp contrast to *Y. pestis*, infectious *V. cholerae* strains are in the essence just biotypes of marine free-living/symbiotic vibrio that acquired virulence determinants in the course of lateral gene transfer mediated by filamentous bacteriophages; in fact, the majority of environmental isolates of *V. cholerae* are non-pathogenic [24]. In its environmental phase, *V. cholerae* inhabits tidal estuaries and riverine systems, where

it is surviving periodical swings of salinity and pH (see the above Sect. 17.2.3). In these aquatic environments, *V. cholerae* is often associated with various members of fito- and zooplankton, plants, algae, fish and shellfish [24]. When ingested with contaminated food or water, *V. cholerae* colonizes the small intestine where it produces the potent enterotoxin called cholera toxin, or CT. The action of CT disturbs the electrolyte balance in the gut and causes cholera, the most severe of many diarrheal diseases known, characterized by significant morbidity and mortality, especially in children in developing countries. In the course of profuse diarrhea, large numbers of bacteria are shed into environment (in the typical for cholera patients "rice water" stool), thus completing the "open" life cycle of *V. cholerae*.

17.3.2 Roles of Na^+ Circulation in Virulence of V. cholerae and Y. pestis

Despite of the significant differences in biology, one of the most peculiar common features of both *Y. pestis* and *V. cholerae* is stringent dependence of their physiology and virulence upon alkali/alkaline earth cations, primarily Na^+ and Ca^{2+} [16, 23, 25, 26]. Although pathways of circulation of Na^+ and Ca^{2+} in both bacteria are intertwined, in this communication we shall focus on roles of sodium ions.

V. cholerae has a strict Na^+ requirement for its growth, with the optimum at ~2 % NaCl [27]. Ability to survive in saline aquatic habitats is believed to play a key role in the persistence of cholera, defining it as "emerging and re-emerging infection" [16]. Interestingly, efficient colonization of the human intestines by *V. cholerae* requires secretion of the CT, which, in particular, elevates the concentration of sodium ion in alkaline intestinal lumen, probably aiming at the imitation of comfortable for the pathogen sea-water conditions, where the efficiency of transmembrane Na^+ circulation is high [28].

Significantly, it has been experimentally demonstrated that changes in the transmembrane circulation of Na^+ in *V. cholerae* affect the expression of a major transcriptional regulator of pathogenicity genes, ToxT, which governs synthesis of virulence factors such as cholera toxin and toxin co-regulated pili [25]. The molecular mechanism of this impact remains unknown, but one likely scenario is that one of membrane Na^+ transporting proteins serves as a SMF sensor, which transmits information about the current SMF level to the ToxT promoter [29]. Thus, both general physiology and regulation of virulence in *V. cholerae* seem to be dependent on the operation of the Na^+ cycle.

The active Na^+ cycle, especially secondary Na^+/H^+ exchange, is expected to be critical also for the survival of *Y. pestis* in the host and the development of the infectious process. Indeed, it is well established that, in order to assure the dissemination via the arthropod vector, population of *Y. pestis* must achieve a remarkably high concentration of app. 10^8 bacteria per ml of blood of mammalian host

[23, 30]. This means that *Y. pestis* cells are thriving in a relatively saline, sea-like environment. Blood plasma normally contains from 135 to 145 mM of Na^+; such level of sodium is lethal for bacterial cytoplasm [31]. On the other hand, electrogenic Na^+/H^+ antiporters of the NhaA type are regarded as major sodium-extruding systems in enterobacteria for their ability to create and maintain large concentration gradients of Na^+ on the bacterial membrane at the expense of the PMF (see [31–33] and Fig. 17.1). It could therefore be expected that elimination of Yp-NhaA would be detrimental for survival of *Y. pestis* in blood and seriously affect (if not prevent) the development of infection.

Again, in a striking similarity to *V. cholerae*, not only survival *per see*, but also the biochemical machinery of virulence in *Y. pestis* seems to be dependent on the Na^+ circulation. *Y. pestis* uses the elaborate type III secretion system (T3SS) to inject a set of proteinaceous virulence factors (collectively known as Yops) directly into the cytoplasm of the target host cells that are in contact with the bacteria [34]. Yops modulate cellular functions to shut down key defensive mechanisms, such as cytokine production and phagocytosis, resulting in total suppression of the host's inflammatory response [35]. Importance of the T3SS for pathogenicity of *Y. pestis* could be well illustrated by the fact that the T3SS-less mutants are completely avirulent [36]. Although the T3SS-mediated substrate translocation is believed to be directly energized by the PMF rather than SMF [37], it has recently been found that operation of the T3SS system renders cells of *Y. pestis* hypersensitive to Na^+, apparently elevating the cytoplasmic concentration of sodium [26, 38, 39]. In fact, careful analysis of the experimental data reported in [37] supports the idea that in yersinia T3SS could mediate antiport of Yops with Na^+ rather than H^+ (PD, unpublished). Regardless the exact molecular mechanism underlying the T3SS-dependent Na^+ sensitivity, however, it seems certain that the elimination of functional Na^+/H^+ exchange would make the activation of T3SS suicidal for *Y. pestis*.

In accordance with the important roles of Na^+ circulation in physiology of *Y. pestis* and *V. cholerae*, the genomes of both organisms encode all essential elements of the Na^+ cycle, excluding only Na^+-motive ATPsynthase [12, 13]. One could therefore hope that manipulating the sodium homeostasis (e.g., through the inhibition of corresponding transport systems) will provide effective means for disruption of the infectious cycle of these dangerous pathogens, eventually yielding effective anti-bacterial remedies with completely new mechanism of action.

17.4 Critical Review of NQR and NhaA/B Pair as Prospective Drug Targets

Both NQR pumps and Na^+/H^+ antiporters of NhaA-, NhaB-type are the core elements of the Na^+ cycle of a very high occurrence in pathogens [13], which makes them the first-rate prospective targets for the development of new antimicrobials.

These also are two classes of Na^+-transporting enzymes for which specific inhibitors have already been reported.

17.4.1 Physiology of Na^+-Translocating NADH:Ubiquinone Oxidoreductase (NQR)

An additional (and very important) feature of NQR as a prospective drug target stems from its phylogeny. Indeed, NQR has no homologues not only in the respiratory chain of human mitochondria, but also in membranes of the benign bacterial species comprising normal microflora; in fact, practically all NQR-possessing species characterized so far are either marine or blood-borne bacteria [13, 17]. This means that any reasonably specific inhibitor of NQR would pose little or no danger of unwanted side effects when administered to the patients. The efficacy of anti-NQR remedy will be dependent on a role that NQR plays in the overall energetics of a given pathogen.

In general, NQR is not essential for survival of marine vibrios (see [16] and references therein). However, *V. cholerae* mutants, in which NQR operon has been interrupted by transposon mutagenesis, displayed inhibition of growth at low and high concentrations of NaCl, suggesting an involvement of NQR in the cellular Na^+ homeostasis [25, 40]. Moreover, NQR might be involved into regulation of the ToxT regulon in *V. cholerae* more directly. Indeed, it has been shown that *nqr* mutations, as well as NQR inhibitors increase the expression of genes encoding major ToxT-dependent virulence determinants, including CT and toxin co-regulated pili [25].

Unlike *V. cholerae*, respiratory electron-transfer chain of *Y. pestis* contains not only the Na^+-translocating NQR, but also a regular H^+-translocating NADH:ubiquinone oxidoreductase [13]. Both enzymes are full functional analogs, feeding the electrons taken from NADH into the respiratory chain at the level of quinones. Thus NQR is expected to be of lesser significance for the survival of *Y. pestis* compared to *V. cholerae*. However, the situation might be very different in other NQR-possessing pathogens. Unexpected immunogenic properties of NqrA from *A. pleuropneumoniae* and *P. gingivalis* were already mentioned above (see Sect. 17.2.4). Of course, the maximal impact of inhibiting the NQR could be expected in pathogens where NQR is the only primary ion pump, as in *Clostridium difficile* [13] or *Treponema denticola* (H^+-motive nicotinamide nucleotide transhydrogenases accompanying NQR in a number of species normally serve as a source of NADPH and consume the PMF rather than generate it), or at least a major primary generator of SMF, as it seems to be the case in *Haemophilus influenzae*, *Actinobacillus actinomycetemcomitans*, or chlamydial species [13, 14]. The latter case looks especially promising, because chlamydiae, being obligate intracellular parasites, are energetic analogs of mitochondria, and the blockage of their respiratory electron transfer as early as at the first coupling site should have devastating consequences (see [14] for the details of chlamydial energetics in the context of their pathogenicity).

17.4.2 Catalytic and Structural Properties of NQR

Form the functional point of view, NQR is a unique redox-driven Na^+ pump providing the gateway for electrons into the respiratory chain that is alternative to the H^+-motive NADH:ubiquinone oxidoreductase. The latter has a twice higher efficiency of energy conservation, displaying the ratio of translocated ions per electron, $H^+/e^- = 2$, compared to the $Na^+/e^- = 1$ measured for NQR [16, 41]. NQR takes reducing equivalents from NADH and donates them to the membrane quinone pool. The energy of this redox reaction is used to pump Na^+ from the inner to the outer side of the membrane thus building the SMF (primarily in the form of $\Delta\psi$, "minus" in the cytoplasm), which can then be used for all sorts of metabolic work:

$$NADH + H^+_{in} + Q + 2Na^+_{in} \rightarrow NAD^+ + QH_2 + 2Na^+_{out}$$

Typical NQR is a hetero-oligomer composed of six subunits. Of them, NqrB, NqrD and NqrE are hydrophobic and form intramembrane core of the enzyme; three others, NqrA, NqrC and NqrF, are more hydrophilic and protrude into the cytoplasm [16, 42]. Enzyme contains FeS center and two cofactors (FAD and FMN) that participate in the intramolecular electron transfer [41, 42]. While NqrA,B,C,D, and E subunits have no homologues among proteins with known functions, NqrF looks like an evolutionary product of a "fusion" of ferredoxin to ferredoxin:$NADP^+$ oxidoreductase of plant origin [42]. It contains the NADH-binding site, as well as FAD and the FeS center; purified NqrF alone is able to catalyze the Na^+-independent NADH dehydrogenase reaction, i.e. oxidation of NADH by an appropriate electron acceptor, such as water-soluble quinines or ferricyanide [43]. *In vivo*, the NADH dehydrogenase (NqrF) transfers two electrons taken from NADH to the quinone reductase, formed by a trio of integral membrane subunits NqrB, NqrD and NqrE [16, 41–43]. Reduction of ubiquinone by the quinone reductase module of NQR is Na^+-dependent, so that this reaction virtually does not occur in Na^+-free media; its K_m for Na^+ is in the low mM range (reviewed in [42]). Mechanism of coupling of the scalar reaction of ubiquinol formation to the vectorial translocation of Na^+ ions by NqrBDE module is still not quite clear. It could be based on the formation of an unusually stable anionic flavosemiquinone in the middle of the membrane and its transient neutralisation by the Na^+ cation captured from the cytoplasm (see [42] for an interesting discussion).

17.4.3 Known NQR Inhibitors

The NqrF-dependent NADH dehydrogenase reaction is sensitive to heavy metals (Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}) [44] and especially Ag^+, which is active in nanomolar concentrations [45, 46]. Using purified NQR, Ag^+ was shown to irreversibly bind to the NqrF subunit, denaturing it and causing the loss of its FAD cofactor [47].

Half-maximal inhibition of the enzyme activity was attained at concentrations between 0.5 and 2 nM Ag^+, making NQR one of the most vulnerable targets of Ag^+ ions known [47]. However, studying the mechanism of bactericidal effect of silver ions on *V. cholerae*, we have found that low-micromolar concentrations of Ag^+ ions had strong bactericidal effect on both wild-type *V. cholerae* O395N1 and on its isogenic NQR-negative mutant growing in batch culture: addition of 1.25 μM $AgNO_3$ killed the cells of both strains [48]. Experiments with inside-out subbacterial vesicles derived from both strains showed that, irrespectively of the presence of NQR, addition of Ag^+ ions provoked total collapse of the respiration-generated PMF on the membrane [48]. Therefore, NQR apparently is not the only target for Ag^+ ions that is present in the membrane of *V. cholerae*, and denaturation of NqrF does not affect Ag^+-induced H^+ leakage in the membrane of *V. cholerae*, which eventually results in total dissipation of all transmembrane ion gradients and inevitable cell death [48]. Although these results are in accord with abovementioned observations about NQR being non-essential for survival of marine vibrios in cultures (Sect. 17.4.1), they do not guarantee that the elimination of functional NQR would not affect infectivity of *V. cholerae in situ*.

Continuing the line of reasoning that the maximal impact of inhibiting the NQR could be expected in pathogens where NQR is the only (or at least the major) primary ion pump (see Sect. 17.4.1), one may note here that silver nitrate had noticeably strong antimicrobial activity against some periodontal pathogens that have *nqr* gene homologs in their genome, such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* [49]. Furthermore, Ag^+ ions strongly affected survival of *Legionalla pneumophila* but had much lesser toxicity for *Mycobacteria avium* [50], which correlates with the presence of *nqrF* gene homolog in the genome of *L. pneumophila*, but not *M. avium*. These observations further support the idea of NQR as a prospective antibiotic target.

Although it looks as ultra-low concentrations of silver are indeed targeting NqrF *in vivo*, Ag^+ obviously cannot be considered a *specific* inhibitor of NQR. Two other known inhibitors of NQR arrest the Na^+-dependent reduction of ubiquinone thus targeting the quinone reductase module of the enzyme (NqrBDE). The first, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) is a natural anti-microbial agent produced by *Pseudomonas aeruginosa* and showing the activity against Gram-positive species such as *Staphylococcus aureus* [51]. HQNO has long been known as the respiration inhibitor arresting the electron transfer through the cytochrome bc_1 segment of the mitochondrial respiratory chain [52]. Being an analog of quinone, HQNO also mediates ΔpH-driven transmembrane proton transport when added at higher concentrations (3–10 nmol/mg of mitochondrial protein), thus acting as an uncoupling permeable weak acid [52]. Later it has been shown that HQNO also binds to the hydrophobic NqrB subunit and non-competitively inhibits the reduction of quinone Q-1 by the isolated NQR with K_i = 300 pM [47]. The abilities of HQNO to block mitochondrial respiration and to cause the transmembrane H^+ leakage somewhat diminish its potential as a platform for the development of anti-NQR drugs. Another inhibitor of NQR, korormicin, was isolated from the marine bacterium,

Pseudoalteromonas sp. F-420 and showed the activity against a number of marine Gram-negative bacteria but not "against terrestrial microorganisms" [53]. It turned out that korormicin is a powerful inhibitor of NQR. Like HQNO, it acts as a non-competitive inhibitor of the NQR-catalyzed reduction of quinones [47]. In the preparations of isolated enzyme reacting with Q-1, korormicin showed K_i of app. 80 pM and acted mutually exclusive to HQNO, suggesting very close binding sites for both inhibitors on NqrB [47]. Mutation of a single amino acid residue, Gly140 → Val140, in the middle of one of the transmembrane α-helical segments of NqrB, increases the resistance of enzyme to korormicin (but not to HQNO) 10^5-fold, suggesting that Gly140 might be a part of the korormicin-binding site [54]. Importantly, korormicin proved to be a specific inhibitor of NQR, as it had no effect on Na^+ -independent NADH oxidase (NADH:menadione reductase) activity [47]. Unfortunately, korormicin remains an exotic compound not available commercially, and data on its pharmacological properties are scarce.

17.4.4 Na^+/H^+ Antiporters of NhaA and NhaB Types

$Na^+(Li^+)/H^+$ antiporters from *Escherichia coli*, Ec-NhaA and Ec-NhaB, represent a combination of secondary Na^+ pumps that is standard for enterobacteria, including *V. cholerae* and many other pathogens [13, 32]. Of these two ion transporters, NhaA is the major antiporter of *E. coli* and other enterobacteria [32]. Ec-NhaA has been studied most extensively in respect of its function and structure (see [31–33] and references therein). It is indispensable for the adaptation to high salinity, resistance to Li^+ ions and growth at alkaline pH in the presence of Na^+, while NhaB seems to be an auxiliary system, which is only necessary for growth on Na^+-symported substrates such as glutamate and proline under conditions in which NhaA is not active or *nha*A gene is not expressed, i.e. in acidic and Na^+-poor media [55].

Expression of *nha*A in *E. coli* is governed by two promoters, P1 and P2, and is subject of a complex regulation at the level of transcription, including the induction from P1 by cytoplasmic Na^+ and Li^+ ions through a regulator NhaR during the exponential phase of growth and the activation of P2 by σ^S in stationary-phase cells [56]. NhaR-dependent regulation was also reported for NhaA in *V. cholerae* [57]. Mutants deficient in both antiporters are extremely sensitive to Na^+ and Li^+, but at low concentrations of these alkali cations in the medium it grows as well as the wild type throughout the whole pH range [31].

The Ec-NhaA protein was purified in its functional form and reconstituted into proteoliposomes, where its activity was found to be strongly dependent on pH, with the V_{max} increasing by about 2,000-fold at pH 8.5 relative to neutral pH [10]. Turnover rate of Ec-NhaA in its pH optimum is amazingly high: 10^5 min^{-1} [31], defining NhaA as one of the fastest transporters ever described. Indeed, such turnover numbers are typical for ion channels rather than for *bona fide* secondary transporters with characteristic turnover rates close to ~10^3 min^{-1} [31, 33]. Such fast turnover is apparently due to relatively small conformational changes in the course of the NhaA catalytic cycle proposed from analysis of the structure of Ec-NhaA determined at the 3.45 Å resolution (see [33]

for the excellent review). According to the current alternate-accessibility model, all substrate cations (including protons) compete for a single ion-binding site located in the middle of lipid bilayer [33]. During the catalytic cycle, NhaA molecule swiftly alternates between two conformations that are open to the opposite sides of the membrane [33]. Movements of atomic groups required for this re-orientation are small enough to allow for high turnover rates measured in experiments.

The reconstituted Ec-NhaA was found to be electrogenic, showing the stoichiometry of $2H^+/Na^+$ as measured by kinetic and thermodynamic methods [10, 31]. Measurements of activity in inside-out sub-bacterial vesicles containing heterologously expressed NhaA proteins from *V. cholerae* and *Y. pestis* were performed in our laboratory ([58] and unpublished data). They showed that these proteins, Vc-NhaA and Yp-NhaA, also are electrogenic, exchanging more than 1 H^+ per each Na^+ ion, and have pH profiles of activity nearly identical to that of Ec-NhaA, with no activity at pH 6.5 and maximal activity near pH 8.5–9.0. One could therefore assume that the NhaA isoforms from different species have similar enzymological features including sensitivity to inhibitors. It should be noted here that Ec-NhaB also is electrogenic with a stoichiometry of $3H^+/2Na^+$; its apparent K_m to Na^+ decreases with pH (from ~16.7 mM at pH 7.2 to ~1.5 mM at pH 8.5) without changes in V_{max} [55]. Being electrogenic, NhaA and NhaB, each within its own pH-optimum of activity, efficiently convert the $\Delta\psi$ generated by primary ion pumps (H^+-motive and Na^+-motive alike) into ΔpNa, which could be directly used to energize Na^+-dependent motility and/or Na^+-symports as well as ATP synthesis; in addition, NhaA, maximally active in alkaline media, prevents fatal alkalinization of the cell interior, allowing $2H^+$ into the cytoplasm per each expelled Na^+ ion (Fig. 17.1).

Working in concert, NhaA and NhaB antiporters ensure the maintenance of sufficiently high ΔpNa over the wide range of external pH. Noticeably, despite of the presence of extended set of at least seven different Na^+/H^+ antiporters in *V. cholerae*, inactivation of the gene encoding Vc-NhaA nevertheless resulted in some inhibition of growth in Li^+-rich media at pH 8.5 [59]. Furthermore, using a *nhaA′-lacZ* transcriptional fusion it has been shown that both Na^+ and Li^+ induce its transcription in *V. cholerae* [59]. Fortunately, *V. cholerae*, whose membrane is full of different Na^+/H^+ exchangers, is a rare exception among other pathogens that usually inhabit niches with much less variable pH and salinity. The maximal impact of inactivation of a given Na^+/H^+ antiporter should be expected in pathogens where the secondary Na^+-extruding machinery is less elaborated, such as *T. denticola*, *Ch. trachomatis*, *Ch. pneumoniae*, *P. gingivalis*, *Ent. faecalis*, *C. difficile*, and many others (see Table 2 in [13] for more examples). In particular, genome of a dangerous pathogen *Y. pestis* contains four genes encoding putative Na^+/H^+ antiporters: *nhaA*, *nhaB*, *nhaC* and *nhaP* [13]. Of them, Yp-*nha*P seems to encode an ion exchanger of broad specificity with K^+ and, possibly, Ca^{2+} as preferable substrates (C.C. Häse, C. Resch and P. Dibrov, unpublished observation). Remarkably, the double Δ*nha*AΔ*nha*B mutant of *Y. pestis* lost its ability to grow in blood or serum *in vitro* (C.C. Häse, personal communication). Given the role of Na^+ homeostasis in physiology of *Y. pestis* discussed above in Sect. 17.3.2, this result is not entirely unexpected; so one may hope that in the future inhibition of NhaA/B pair will provide a new way of treatment of the blood-born infections.

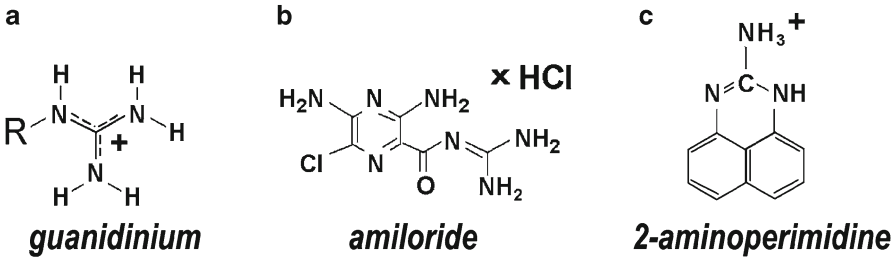

Fig. 17.2 Structures of R-substituted guanidinium ion (**a**), amiloride (**b**) and 2-aminoperimidine (**c**). Only one of two possible tautomeric forms of 2-AP (the $=NH_2^+$form and the $–NH_3^+$ form) is shown. See text for further detail

17.4.5 Pharmacology of NhaA and NhaB

Unlike mammalian Na^+/H^+ antiporters belonging to the NHE family, for which an amazing number of competitive inhibitors derived from a popular diuretic drug amiloride have been synthesized over the years (see [58] and cited literature), pharmacology of bacterial Na^+/H^+ antiporters remains virtually undeveloped. Amiloride (Fig. 17.2b) and its derivatives inhibit Na^+/H^+ exchange because of their guanidine group (Fig. 17.2a), which mimics structurally the tri-hydrated Na^+ ion and thus can compete with Na^+ for the cation-binding site of the antiporter (see [58] for discussion). Amiloride itself has been shown to competitively inhibit NhaB with a disappointingly high K_i = 6 μM, as determined using the purified NhaB reconstituted into proteoliposomes [60]. The ability of amiloride to inhibit human NHE-1 antiporter with better K_i of app. 1.6 μM [60] makes this compound somewhat less attractive candidate for a use as antimicrobial remedy, at least without structural modifications that could enhance its specificity and K_i.

An initial screening of amiloride derivatives yielded no inhibitor effective against Ec-NhaA [55, 60]. No specific inhibitor has been reported for any member of the NhaA family until 2005, when we, by serendipity, came across 2-aminoperimidine (2-AP), which turned out to be the first specific inhibitor of NhaA-type antiporters [58]. 2-AP is a product of fusion of guanidine group to the naphthalene moiety (Fig. 17.2c), which at the physiological pH values exists as a cation (2-perimidinylammonium ion) in two possible tautomeric forms (the $=NH_2^+$ form and the $–NH_3^+$ one). 2-AP inhibited, in the competitive manner, the activity of Ec-NhaA in sub-bacterial membrane vesicles with K_i close to 0.9 μM; the same concentrations of 2-AP arrested the $^{22}Na^+/Na^+$ exchange mediated by purified Ec-NhaA incorporated into proteoliposomes [58]. Importantly, when assayed in sub-bacterial vesicles containing functionally expressed Na^+/H^+ antiporters of different types and origins, 2-AP inhibited the activity of Vc-NhaA, but not of Ec-NhaB or Vc-NhaD [58]. Thus, 2-AP seems to be specific for the enterobacterial antiporters of NhaA type. It should be noted here, however, that 2-AP is known as a sulfate precipitant and potent inhibitor of cholinesterases [61]. In addition,

being at the same time a membrane-penetrating cation and a weak amine, 2-AP at high concentrations apparently dissipates both electric and osmotic constituents of the PMF on the membrane [58]. Nevertheless, given enormously extensive chemical libraries of amiloride derivatives that have been accumulated in pharmaceutical industry to date, one may optimistically look forward to screening them for anti-NhaA/B active compounds that would serve as platforms for the development of novel, precisely targeted antimicrobials free from undesirable side effects.

17.5 Conclusion

There are many other types of Na^+-motive transporters that deserve serious attention as potential targets for the future drug development. One could point to Na^+-motive dicarboxylate decarboxylases that are able to generate the SMF in the absence of electron transfer [7, 13]; cation/proton antiporters of NhaP type that in some cases interconnect circulation of Na^+, as well as Ca^{2+} and/or K^+ [62]; Na^+-motive ATPsynthases [12, 13]; or a variety of Na^+-dependent symporters [13].

Although systematic screening and evaluation of the Na^+ cycle constituents as prospective targets for the anti-microbials of the next generation still remains a prospect of indefinite future, it offers unique opportunities of targeting the membrane-bound ion transport systems specific for particular pathogens and disrupting infectious processes in a way that could not apparently be countered by multi-drug resistance pumps – advantage that we cannot afford to ignore much longer at the looming end of the current "era of antibiotics".

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Chapter 18
Natural Antimutagens for Environmental Quality Management and Transition to Ecological Civilization

Urkhan Alakbarov

Abstract Environmental management is critically dependent upon the practice of ecologically friendly "green" technologies and in changing the life styles of human populations to an ecologically friendly mentality. The transition to an ecological minded civilization requires an intense educational process of the public to increase their knowledge of the serious condition of the environment. The presence of a "green" mentality in the population will mitigate the environmental impacts associated with the use of a variety of dangerous chemicals and carcinogens with mutagenic properties. In addition, the potential for some species of plants to be used to mitigate this environmental impact is exciting and very real.

The modern philosophy and practice of environmental management is based on principles for an ecological civilization, implementation of ecologically friendly "green" technologies, modifying human life style and human behavior in an ecologically friendly manner, and the formation of a "green" mentality [1, 2]. Capacity building to enable a transition to an ecological civilization involves many different actions, including formal and informal education on relevant occupational/professional knowledge and skills at both secondary and tertiary levels, as well as increasing the general knowledge and awareness of the people for sustainable development. The implementation of this program is based on knowledge and awareness about existing ecological situation, sources of environmental contamination and degradation, and updated information about the assessment of environmental quality and management methodology. The prevention and mitigation of environmental damage are also based on information about the ecological status of the environment as

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well as the potential to mitigate the consequences of contamination by mobilizing natural biologically active compounds with protective and preventive properties. Therefore, the first steps toward capacity building for development, implementation and monitoring ecological civilization processes are environmental quality assessment and environmental quality management with the mobilization of biologically active compounds from plants and plant mixtures for the prevention and mitigation of environmental impacts [3].

The assessment of environmental quality has shown that the problems associated with environmental degradation and environmental contamination are almost always ecological problems that are a result of previous and ongoing economic activities of the human society and life style. Starting from our early years of civilization until today, the increasing number and density of our population, the processes involved in establishing new cities and villages, the expansion of existing human settlements, the laying of communication lines and constructing roads, the increased transportation have all been associated with the contamination and eventual destruction of our environment. The same negative eco-toxicological effects have resulted from economic projects in the field of industry and agriculture. Exploration and the subsequent development of the extractive and manufacturing industries have contributed to the degradation and contamination of the air, water and soil. All of these factors have lead to an increased health risk for people and a decrease in the biodiversity found in the environment in which we live. Mobilized agrarian technologies have resulted in overuse of agricultural techniques and chemicals which has in turn also initiated the processes of soil degradation, soil erosion and environmental contamination. The presence of residual chemicals in agricultural products and foods, and the existence of genetically modified food products are another ecologically problem. This, in turn, has triggered processes with immediate and long term effects on the loss and degradation of biodiversity, and negative health effects for both current and future generations [4]. Alternatively, increasing our knowledge about biologically active compounds with protective properties represents a different way for addressing this problem. This may be achieved through the breeding of new agricultural plants and animal varieties, the creation of a new generation of foods and food additives, as well as medicines with appropriate characteristics [1, 2, 5].

One of the most important requirements for the planning of environmental management is managing environmental quality through the mobilization of biologically active compounds. This can help to mitigate the scale and level of those changes in the environment which normally occur as a result of naturally ongoing processes or anthropogenic impacts. Human activities also have a significant impact, including that resulting from industrial and agricultural technologies as well as resulting from the natural processes may initiate changes on different levels of biological organization of the nature. These levels involve different scales from the multi-component ecosystem or the biogeocenotic level to a molecular-genetic approach (Fig. 18.1).

Generational and regulated genetic disorders induced by chemical, physical and biological environmental contaminants can also produce pathological processes in all living beings. These result in increases in different human diseases, including cancer and early ageing. The transition to an ecological civilization by implementing

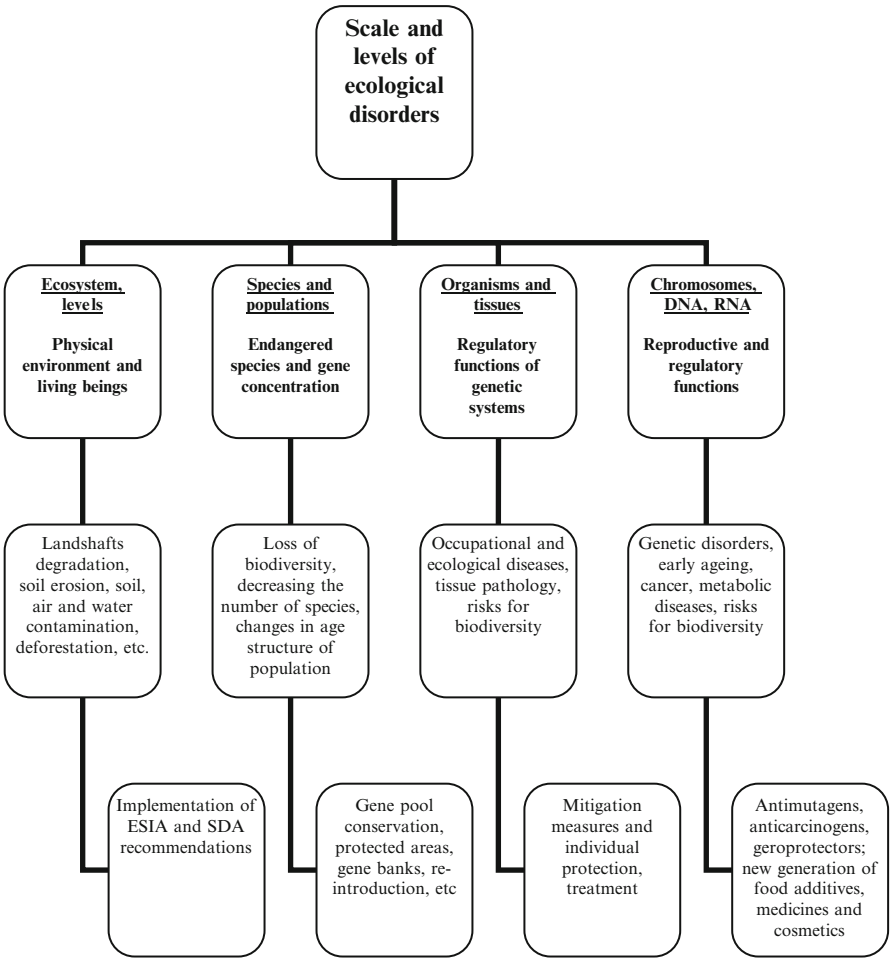

Fig. 18.1 Levels of environmental degradation: classification, assessment and management. *ESIA* Environmental and Social Impact Assessment, *SDA* Sustainable Development Assessment

green technologies in industry, agriculture, construction and transportation are the most effective approaches for decreasing and ultimately preventing the genetic consequences of environmental contamination with chemical and physical xenobiotics (Fig. 18.2). Antimutagenesis is another important element of the planning and management strategies to implement an ecologically stable civilization (ecocivil) and is considered to be one of the most feasible approaches to the prevention and reduction of genetic pathology induced by chemical, physical xenobiotics and biological environmental contaminants. A comparative assessment of the efficiency of the synthetic and natural antimutagens has shown that natural inhibitors of spontaneous and induced genetically disorders, including those of plant origin are more effective than synthetic antimutagens [2, 3].

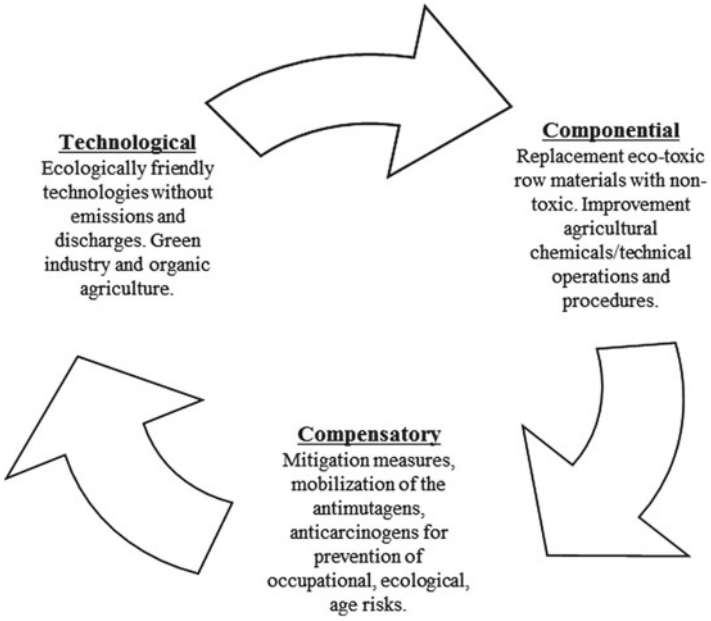

Fig. 18.2 Environmental risks management on technologically, componential and compensational levels

In order to transition to an ecologically friendly civilization, effective mobilization of green technologies and implementation of a green mentality and green behavior can be achieved in the future [2]. Today, addressing the genetic consequences of environmental contamination by xenobiotics or natural contaminants can be achieved through the implementation of different principles and technologies. Decreasing the contamination level and/or replacement of the ecotoxic xenobiotics with non-toxic xenobiotics are the most appropriate way to manage environmental quality (Fig. 18.2).

Full scale implementation of these principles is not a reality at the moment due to complicated engineering and economical problems. Along with an improvement of the environmental quality of technologically and componential levels, the regulation of the resistance of the living beings to the mobilization of different biologically active compounds having antimutagenic, anticarcinogenic and genoprotective properties are different and more realistic possibilities for addressing the ongoing and future consequences of environmental contamination. The biologically active compounds isolated from different plants and their mixtures possess genoprotective properties against various environmental mutagens and oncogens. Mobilization of these compounds is the most appropriate way to regulate genetic resistance.

The influence of antimutagens on mutation has been studied on various objects. The antimutagens have been obtained from different plant groups belonging to the *Glycyrrhiza*, *Cydonia*, *Diospyros*, *Punica*, *Morus* and others. The individual

Table 18.1 Genoprotective effects of some plant products and their mixtures

Species	Products	Eco-toxicants	Test objects	Effects
Clycyrrhiza glabra	Extracts from roots	γ-rays, nitrosocompounds, ageing	Plants, mammals	Antimutagenic, geroprotective
Fagus orientalis	Oil from fruits and leaves	γ-rays, X-rays, nitrosocompounds, ageing, pesticides	Plants, mammals	Antimutagenic, geroprotective
Olea europea	Oil	γ-rays	Plants, mammals	Radioprotective
Fagus orientalis + *Olea europea*	Oils, mixture	Nitrosocompounds, ageing	Plants, mammals, microorganisms	Antimutagenic, geroprotective
Punica granatum	Extracts from fruits	γ-rays, ageing, nitrosocompounds,	Plants, mammals, human cells	Radioprotective, antimutagenic, geroprotective
Morus alba, *Morus nigra*	Extracts from Fruits	γ-rays, X-rays, nitrosocompounds, ageing, fluorine	Plants, mammals	Radioprotective, antimutagenic, geroprotective
Fagus orientalis	Phenols	γ-rays, X-rays, nitrosocompounds, ageing, fluorine	Plants, mammals, human cells	Radioprotective, antimutagenic, geroprotective
Armoracia rusticana	Extracts from roots	γ-rays, ageing, nitrosocompounds	Plants, mammals	Antimutagenic,
Yucca gloriosa	Extracts from leaves and polyphenols	γ-rays, X-rays, nitrosocompounds, ageing, pesticides	Plants, mammals	Radioprotective, antimutagenic, geroprotective
Camelia sinensis	Extracts from leaves	Carcinogens	Human cells	Antimutagenic,
Diospyros kaki	Extracts from fruits	γ-rays, X-rays, nitrosocompounds, ageing, fluorine	Plants, mammals, human cells	Radioprotective, antimutagenic, geroprotective
Cudonia oblonga	Extracts from fruits	X-rays, fluorine nitrosocompounds, ageing	Plants, mammals, human cells	Radioprotective, antimutagenic, geroprotective

Table 18.2 The principal components of ecological civilization

Ecologization	Industry/construction	Implementation of the green technologies in extracting and manufacturing industries. Addressing all problems related with industrial emissions and discharches. Green settlements, green houses constructing and equipping (biofuel, biodegradable materials, green construction technologies, equipments, devices, etc.).
	Agriculture	Development of the organic agriculture, sustainable forestry, fishery, etc.
	Transport	Energy saving, recoverable energy, "zero discharges". Increasing the share of public transportation.
	Education	Mandatory green education on primary, secondary and tertiary levels.
Demography	Number and density of population	Demography and birth rate control, optimization of the population density and settlements sizes.
	Health	Decreasing the risks factors and increasing of the antirisk factors for long and healthy life.
	Quality of life	Natural, economical, political, social environment providing with prosperity any personality.
Consumption	Culture of consumption	Consumption on the level of reasonable needs.
		Life style and consumption patterns for management of any existing and potential risks for long, healthy and creative life. Based on combination of the most updated scientific research with historically proven ethnic/local knowledge and culture. Biodiversity conservation.
		Reduction of the social disparities.

compounds, the sum of the biologically active compounds obtained from these plant species as well as their mixtures decreased the level of mutations and modifications in the cells of plants and animals. The extracts from these plants and their mixtures also decreased the genetic disorders induced by X-rays, gamma-rays, N-methyl-N-nitrosourea, cyclophosphamide, sodium fluoride and other environmental toxicants. The plant products in a complex mixture demonstrated the highest efficiency when the components have been tested separately [3, 6]. The plant products studied and their mixtures also decreased the level of mutability induced during the aging processes. The following plants products possessed the genoprotective properties (Table 18.1) [3, 6].

Transformation to a green economy, along with the development of a corresponding technological environment and the creation of opportunities that would favour a green environment should prepare the society for similar transformations. It is obvious that the effective functioning of a green economy can only be reached in the presence of a corresponding national policy with close cooperation from state structures, a civil society and private business. The principal component of an ecological civilization is presented in the Table 18.2 [2].

Important elements of the ecocivil are the presence of a "green" mentality in the people, relevant professional readiness and general awareness in the population of

the possibilities that will mitigate the environmental impacts associated with the use of antimutagens, anticarcinogens and genoprotectors with genoprotective properties. The sources listed in Table 18.1 and some other species of plants can be used for environmental impact mitigation.

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Part IV
Biotechnological and Therapeutical Aspects of Defense Against Radiological, Chemical and Biological Agents

Chapter 19
Grape Polyphenols Attenuate Psychological Stress

Eugene Y. Brunner and Volodymyr I. Mizin

Abstract The process of learning can be regarded as a model of psychological stress, since any active operator has to cope with the tasks of attentive and quick perception and memorizing of information, to work out an adequate behavior model as well as to implement it in practice. Forty-three students (23 – main group, 20 – control group) took part in the estimation of psychological state, personality characteristics, auditory arbitrary memory, the dynamics of attention processes, attention qualities, speed of sensor-motor reaction and thinking operations. The students of the main group ingested the alcohol-free dietary grape polyphenol concentrate Enoant for 20 days at a daily dose of 35 ml. Statistically significant changes, such as (i) improvement of the activity and mood; (ii) improvement of attention, (iii) increase in thinking productivity and concentration of attention; (iv) quantity of the processed visual information and the rapidity of processing; (v) improvement of the examinees short-term and working memory; (vi) increase of intellectual work capacity; (vii) success in task fulfillment, and (viii) diminishing of incorrect actions were observed only in the main group. The Enoant use led to the positive changes of reflexivity regarding emotional-volitional and activity spheres of examinees. Thus, Enoant use increases the reserve of adaptation to psychological stress and it can be used as a countermeasure against psychological stress in personnel. Enoant may also be successfully employed in the control of psychological stress at periods of alcohol consumption restriction and under the risk of alcohol addiction development.

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19.1 Introduction

During recent decades, a new strategy for the development of novel medicines has been created and consequently there has appeared a number of trends among which adaptive medicine is notable. Within this framework, adaptive medicine studies the capacity for humans to adapt to a changing environment as well as develop the methods and means for increasing such abilities [14, 15]. Factors of adaptation, according to the paradigm of this filed, stimulate hidden body reserves and bring man to a qualitatively new level of health. This field of research is also interesting because it exceeds the limits of conventional medicine, i. e. it unites the interests of physicians and psychologists [1,18].

In addition to adaptative medicine, catastrophe medicine and military medicine are also important today. A vital aspect of military medicine, providing for the effective action of an operator and the healthy recovery of a victim is the resistance to psychological stress. The process of learning can be regarded as a model of psychological stress, since any active operator has to cope with the tasks of attentive and quick perception and memorizing information in order to work out an adequate behavioral model as well as to implement it in practice.

Among the remedies able to stimulate compensatory adaptation is Enoant. Enoant is a food concentrate of grape polyphenols. The medical effects of Enoant treatment were shown recently [2, 4–7, 12, 16, 22]. Almost all the results testify to the positive effects of the Enoant treatment, including the effects connected with the improvement of a healthy feeling and diminution of the stress level. However, almost all the studies were carried out in patients with different diseases. This is why it was considered to be important to carry out a complex psychophysiological study of the influence of the dietary concentrate Enoant in healthy subjects.

The purpose of our research, therefore, was to study the psychological effects of Enoant. Specifically, the objective was to study the influence of Enoant upon the cognitive and personality spheres of students. Thus, the objective of our research was the study of Enoant influence upon certain constituents of cognitive and personality spheres of students from the Crimean University of Humanities under conditions of increased intellectual load. The hypothesis of the research was that Enoant would have a positive influence upon the cognitive and personality spheres of men.

19.2 Materials and Methods

The estimation of the psychological state and personality characteristics was carried out with the parameters of the MMPI test by 133 basic and additional scales [9, 11, 19]. There were 43 students (23 in the main group, 20 in the control group) under observation. The estimation of the type of temperament was carried out using the parameters of the Eysenck test (EPQ) and of the level of aspirations with the help of Schwarzlander's method [20]. The estimation of auditory arbitrary memory was

carried out with the parameters of short- and long-term memory for words (Luria's method "10 words learning"), of short-term memory for figures (Jacobson's method) and of working memory [17]. The dynamics of attention processes was estimated by 17 parameters with the help of the proofreading test based on Landolt's ophthalmologic rings [8, 10].

We also used the "Choice" and "Decision-making" tests for estimation of attention qualities, speed of sensorimotor reaction and thinking operations. The "Choice" test aims at the examination of attention as well as of the speed of the reaction to colour signals of various modalities. The "Decision-making" test examines attention span, thinking operation speed and psychic process stability. Testing with these methods was carried out with the help of the computer programme "PFS". This complex of methods was worked out by Zernavy, Korzunin in and Levich at the Military Medical Academy, St. Petersburg, Russia. Anthropometric and physiological parameters were determined with generally known methods [3].

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The students' group examination was carried out in classrooms before classes. The participants of the research took Enoant daily at the same time: at 9:20 AM after breakfast. The test data were processed with the help of the proprietory computer programme written by Brunner [9, 21], Microsoft Excel and statistic software STATISTICA for Windows (8.0). The difference between the studied parameters was estimated with Student's *t*-test [13].

At the first stage, we fixed the initial data by all the above-mentioned methods. At the second stage, the students of the main group took 35 ml of Enoant daily for 20 days. At the third stage, we carried out the repeated examination of the psychophysiological state of the students of the main and the control groups and analysis of the received results dynamics.

19.3 Results and Discussion

The dynamics of the attention parameters (Table 19.1 and Fig. 19.1) showed that, in general, Enoant use had beneficial influence on the examinees. We observed an increase in the correctly crossed out signs (by 38 %), in productivity (by 24 %), in accuracy (by 11 %), in thinking productivity (by 34 %) and attention productivity (by 27 %), in the volume of processed visual information (by 24 %) and in the speed of information processing (by 54 %), in intellectual work capacity (by 37 %), and in the success of task fulfilment (by 11.3–13.8 %). We also established that the students of the experimental group showed considerable diminution (by 53 %) of incorrect actions during the proofreading test. We must emphasize that after Enoant treatment statistically significant changes were revealed in such parameters as ***Σ, P + O, n, A, E, V, Q, Ua, Ayp***, $\boldsymbol{Us_1}$**-** $\boldsymbol{Us_3}$.

Table 19.1 Dynamics of change in attention parameters after Enoant treatment in comparison to the initial level (in the main group of 23 students)

	Studied parameters	Before Enoant use	After Enoant use	Change (%)
N	Number of signs in the worked out part of the table	323.6 ± 70.2	379.7 ± 78.5	24.0
*Σ**	Number of correctly crossed out signs	102.1 ± 25.9	132.6 ± 29.0	38.2
P	Number of missed signs	13.6 ± 12.8	8.0 ± 4.9	−46.2
O	Number of erroneously crossed out signs	4.1 ± 10.5	1.0 ± 2.0	−74.8
P + *O**	Total number of erroneous actions	17.8 ± 22.1	9.05 ± 4.9	−53.2
*n**	Number of signs that had to be crossed out	115.8 ± 22.8	140.7 ± 28.3	27.7
M	Total number of crossed out signs	106.2 ± 24.7	133.6 ± 28.7	33.3
*A**	Productivity	10.8 ± 2.3	12.7 ± 2.6	24.0
T1	Accuracy	0.9 ± 0.1	0.9 ± 0.0	5.1
T2	Accuracy	0.9 ± 0.1	0.9 ± 0.0	8.9
T3	Accuracy	0.9 ± 0.1	0.9 ± 0.0	10.8
*E**	Thinking productivity	285.0 ± 76.9	358.1 ± 80.4	34.3
K	Attention productivity	71.7 ± 31.5	87.2 ± 7.3	26.7
*V**	Volume of visual information	192.1 ± 41.7	225.4 ± 46.6	24.0
*Q**	Information processing speed	0.5 ± 0.2	0.7 ± 0.2	53.6
*Ua**	Working speed stability	157.6 ± 66.4	134.9 ± 38.0	−26.9
*Ayp**	Intellectual work capacity	8.2 ± 3.3	11.2 ± 6.0	36.9
R	Efficiency of work	94.4 ± 6.7	97.3 ± 1.5	3.3
*Us1**	Success	2.0 ± 0.3	2.2 ± 0.3	11.3
*Us2**	Success	1.9 ± 0.3	2.2 ± 0.3	13.0
*Us3**	Success	1.9 ± 0.3	2.2 ± 0.3	13.8

Note: * $p < 0.05$ vs control

The effect of Enoant use was also observed during the examination of short and long-term memory for words, and for short-term and working auditory memory (Table 19.2, Fig. 19.2). Thus, the quantity of items reproduced by the examinees figures increased by 3 %, whereas the examinees' working memory increased by 6 %. At the same time, the volume of short-term and long-term memory for words fell by 3 and 0.2 % respectively. However, applying Student's *t*-test to the memory parameters did not reveal any statistically significant changes in comparison to the initial levels. Thus, the Enoant use led to positive tendencies in operating figures and an insignificant diminution of the volume of memory for words, which was within the frames of permissible norm.

An examination of the speed of sensorimotor reaction (Table 19.3, Fig. 19.3) to colour stimuli of various modalities as well as an examination of the speed of decision-making showed that the students of the main group displayed an increase not only of work efficiency, but also of incorrect actions, though we could reveal no statistically significant changes. This pattern might point to a tendency for the deterioration of sensorimotor parameters and a diminution of critical attitude.

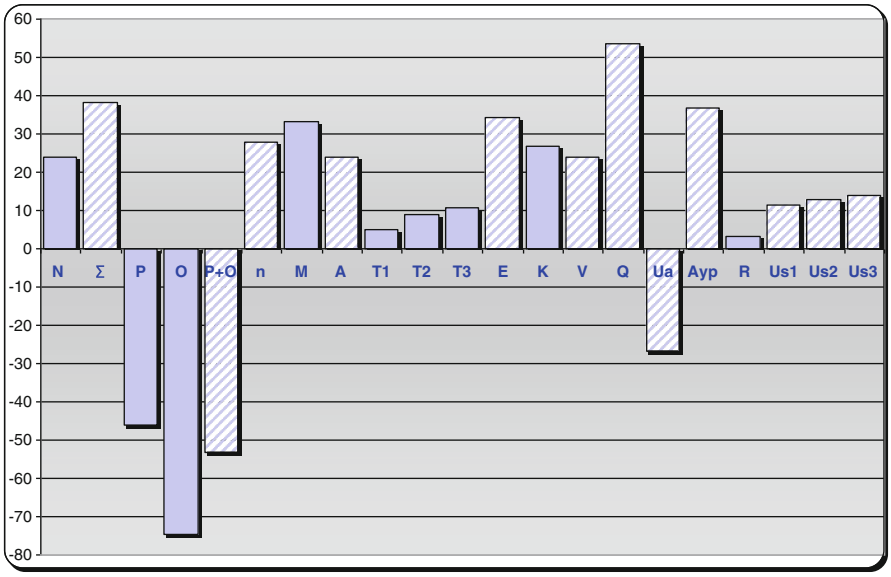

Fig. 19.1 Changes in attention parameters in 23 students after Enoant use in comparison to the initial levels. In the vertical axis, the changes of parameters in % are shown; in the horizontal axis, the names of the parameters as in Table 19.1 are shown. The parameters, which show statistically significant changes ($p < 0.05$) compared to the initial level, are indicated with *hatched bars*

Table 19.2 Dynamics of change in memory parameters after Enoant use in comparison to the initial level (in the main group of 23 students)

Studied parameters		Before Enoant use	After Enoant use	Change (%)
Mem_Lur_1	Short-term auditory memory for words (Luria's method "10 words learning")	6.7 ± 1.6	6.4 ± 1.4	−3.4
Mem_Lur_D	Long-term auditory memory for words (Luria's method "10 words learning")	9.5 ± 1.1	9.5 ± 1.0	−0.2
Mem_Dg	Short-term auditory memory for figures (Jacobson's method)	7.1 ± 0.9	7.1 ± 0.7	3.1
Mem_Oper	Working auditory memory for figures	35.8 ± 4.8	37.0 ± 3.7	6.4

The application of our programme to the processing and interpretation of the MMPI testing result allowed us to reveal changes in the interpretation of mean profiles by 13 basic (Figs. 19.4 and 19.5) and 118 additional scales (data are not shown for brevity) before and after Enoant use.

The dynamics of the MMPI 13 basic scales showed the normalization of the profile after Enoant use in men (Fig. 19.4) and in women (Fig. 19.5). Application of a Student's *t*-test showed statistically significant change in average data of all the scales of the basic profile in male examinees after the treatment (Table 19.4 and

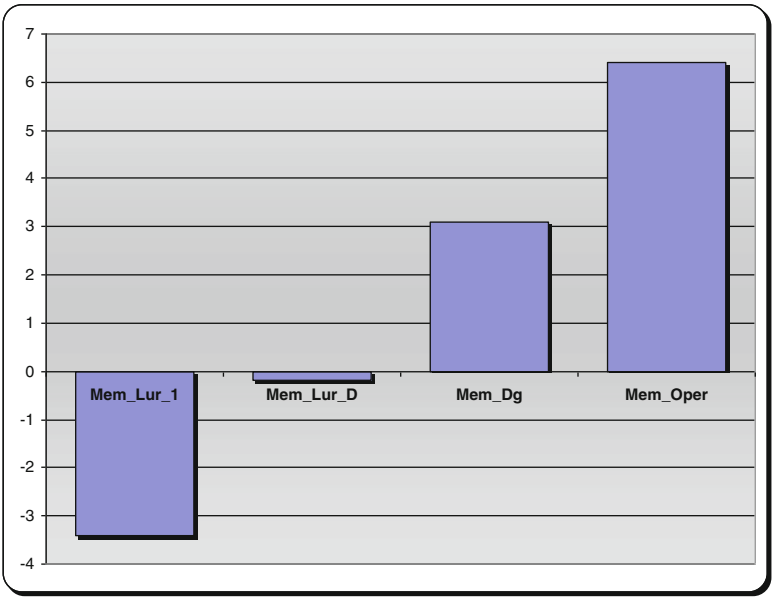

Fig. 19.2 Changes in memory parameters in 23 students after Enoant use in comparison to the initial level. In the vertical axis, the changes of a parameter in % are shown; in the horizontal axis, the names of the parameters as in Table 19.2 are shown

Table 19.3 Dynamics of change in parameters of speed of the reaction to signals of various modalities after Enoant use in comparison to the initial level (in the main group of 23 students)

Studied parameters		Before Enoant use	After Enoant use	Change (%)
Mean_selection	Speed of the reaction to colour signal (the "Choice" test)	179.2 ± 29.8	188.6 ± 26.0	5.3
Mean_selection_ERR	Number of errors during the reaction to colour signal (the "Choice" test)	10.2 ± 9.1	10.9 ± 15.0	5.9
Mean_solution	Speed of the reaction during solving mathematical problems (the "Decision-making" test)	101.3 ± 29.5	112.2 ± 26.7	10.7
Mean_solution_ERR	Number of errors during solving mathematical problems (the "Decision-making" test)	50.7 ± 14.7	56.1 ± 13.4	13.5

Fig. 19.4). The most significant increase was observed in such parameters as "Depression" (scale 2, by 10.9 %), "Hysteria" (scale 3, by 9.2 %), and the most significant reduction – in such parameters as "Defensiveness" (scale K, by 29.3 %), "Anxiety" (scale 7, by 33.1 %) and "Individualism" (scale 8, by 33.4 %).

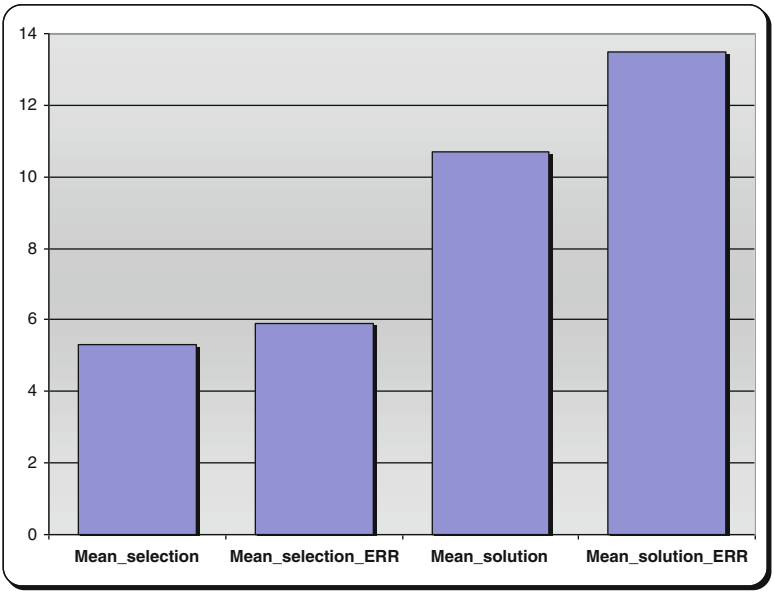

Fig. 19.3 Change in parameters of speed of the reaction to signals of various modalities in 23 students after Enoant use in comparison to the initial level. In the vertical axis, the changes of a parameter in % are shown; in the horizontal axis, the names of the parameters as in Table 19.3 are shown

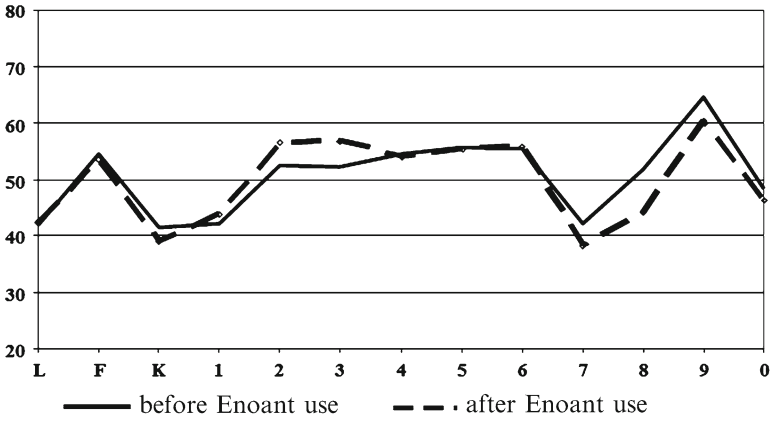

Fig. 19.4 Mean basic MMPI profile in 7 male students before and after Enoant treatment. T-scores are shown in the vertical axis, whereas MMPI scales are presented in the horizontal axis

As in males, we also observed statistically significant changes in practically all of the scales of the MMPI basic profiles in females (Table 19.5 and Fig. 19.5). There were no statistically significant changes only in the "Lie" scale. The most significant increase was observed in such scales as "Hysteria" (scale 3, by 4.5 %), "Social

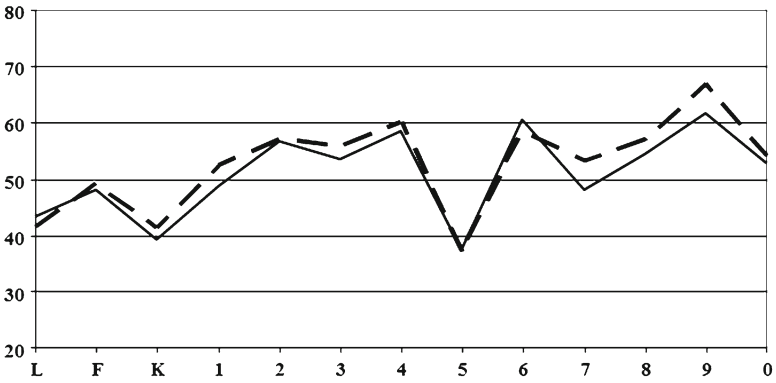

Fig. 19.5 Mean basic MMPI profile in 17 female students before and after Enoant use. T-scores are shown in the vertical axis, whereas MMPI scales are presented in the horizontal axis

Table 19.4 Change in mean values of T-scores (MMPI test) in 7 male students after Enoant use in comparison to the initial level

Scale		Student's *t*-test	Change (%)
L	Lie	t =2.9	1.2
F	Reliability	t =4.5	−10.7
K	Defensiveness	t =3.5	−29.3
1	Hypochondriasis	t =4.7	−14.7
2	Depression	t =4.4	10.9
3	Hysteria	t =4.2	9.1
4	Impulsiveness	t =6.8	−12.6
5	Masculinity/Femininity	t =4.0	−0.5
6	Paranoia	t =4.0	0.8
7	Anxiety	t =6.9	−33.1
8	Individualism	t =7.6	−33.3
9	Optimism	t =3.2	−12.8
0	Social Introversion	t =3.9	−3.6

Introversion" (scale 0, by 4.3 %) and the most significant decrease was found in such scales as "Reliability" (scale F, by 14.6 %), "Defensiveness" (scale K, by 16.6 %), "Masculinity/Femininity" (scale 5, by 6.3 %), "Anxiety" (scale 7, by 7.2 %), and "Individualism" (scale 8, by 9.3 %).

A decrease in the scales of "Depression", "Optimism", "Social Introversion" and "Hysteria" as well as strengthening in the scales of "Masculinity/Femininity", "Anxiety" and "Individualism" can indicate not only certain changes in the behaviors of the women, but also a rise of libido power, i.e. sexual activity. This conclusion was confirmed in private talks with the women who took part in the research.

The analysis and interpretations of the data showed that the Enoant use in males led to active life, higher resistance to stress, better temper, and good adaptation to various environments. The examinees revealed male behavior style, i.e. diminution of the

Table 19.5 Changes in mean values of T-scores (MMPI test) in 17 female students after Enoant use in comparison to the initial level

Scale		Student's *t*-test	Change (%)
F	Lie	t =2.5	−14.6
K	Reliability	t =2.4	−16.6
1	Defensiveness	t =4.6	−2.8
2	Hypochondriasis	t =3.6	1.1
3	Depression	t =4.8	4.5
4	Hysteria	t =4.0	−5.4
5	Impulsiveness	t =3.7	−6.3
6	Masculinity/Femininity	t =3.9	−3.9
7	Paranoia	t =4.5	−7.2
8	Anxiety	t =5.5	−9.2
9	Individualism	t =3.4	1.8
0	Optimism	t =2.4	4.3

anxiety and timidity, diminution of the ability for compassion, non-conformism of personality sets, functional fixity, appearance of brute and rigid behavior, appearance of rather cynical view of life, soberness of mind, and a practical and rational approach to solving problems. Enoant use in the examinees revealed such latent features as intellectual banality, poor fantasy (imagination), lack of originality, and an inability to use a non-standard approach to solving problems.

At the same time in female examinees after Enoant use, we observed positive changes in their attitude towards people, such as appearance of flexibility and of lack of rancour, unforgiveness and bearing a grudge. Their level of sensitivity to therapy increased considerably. Enoant use let us reveal such emotional behavior patterns as difficulty in keeping in the emotional reactions, which, however, are still under control, for the emotional manifestation is thought to be redundant and inappropriate. To analyze in detail the observed changes and to diagnose their reasons, we conducted a content-analysis of the MMPI test statements, which changed their sign to opposite. The results are shown in Tables 19.6 and 19.7.

As can be seen from Table 19.6, the male examinees showed a tendency to control their emotional state to larger extent or, in other words, the changes in their emotional-volitional sphere (e.g. statement 22) had shown more realistic views. This would be indicative of the processes connected with the reflection of their own behavior (statements 141, 327, 504, 509, 537), state (statement 439), attitude towards opposite sex (statement 435), realization of certain traits of their own character (statement 201) and abilities (statements 464, 465), as well as the behavior of other people (statement 117).

Similar to males, the female examinees also showed certain changes in their answers to the MMPI test statements after Enoant use. Notwithstanding certain ambivalence in answers, in general, we also observed a positive influence of Enoant directed, in the first place, towards the processes connected with the reflection and the level of control over their emotional state (statements 27, 170, 299, 341, 381), control over their actions (statements 343, 477), and a tolerance towards other people

Table 19.6 Changes in answers to MMPI statements in 7 male students before and after Enoant use

No.	Answer before Enoant use	Answer after Enoant use	Statement
22	T	F	At times I have fits of laughing and crying that I cannot control
117	T	F	Most people are honest chiefly because they are afraid of being caught
141	T	F	My conduct is largely controlled by the behavior of those around me
201	T	F	I wish I were not so shy
327	T	F	My parents often made me obey even when I thought it was unreasonable
435	T	F	I would prefer to work with women
439	F	T	It makes me nervous to have to wait
464	F	T	I have no ability to see visions
465	F	T	I have several times had a change of heart about my lifework
504	T	F	I do not try to cover up my poor opinion or contempt of people so that they won't know how I feel
509	F	T	I sometimes find it hard to stick up for my rights because I am so reserved
537	T	F	I would like to hunt lions or tigers

Note: *T* True, *F* False, No = identification of statement according to Sobchik modification of MMPI questionnaire

Table 19.7 Changes in answers to MMPI statements in 17 female students before and after Enoant use

No.	Answer before Enoant use	Answer after Enoant use	Statement
27	T	F	Evil spirits possess me at times
46	F	T	My judgment is better than it ever was
73	F	T	I am an important person
170	T	F	What others say about me does not bother me
271	F	T	I do not blame a person for deceiving people who leave themselves open to deception
299	T	F	I think that I feel more intensely than most people do
341	T	F	At times I hear so well that it bothers me
343	T	F	I usually have to stop and think before I act, even in small matters
381	F	T	I am often said to be hotheaded
477	T	F	If I were in trouble with several friends who were as guilty as I was, I would rather take the whole blame than give them away
557	F	T	I would like the work of a secretary

Note: see Table 19.6

(statement 271). Notably, the appearance of positive self-appraisal (statement 46) up to its overstating (statement 73) that points to selectivity of the processes of reflection and self-reflection was observed.

The content analysis shows that Enoant use has led to positive changes in examinees' reflection in their emotional-volitional and activity spheres. Thus, we can conclude that the dietary grape concentrate Enoant exerted a beneficial influence upon the psycho-emotional state of the examinees and its application let us to receive more accurate data about latent features of personality and intellectual activity. Enoant use increases an adaptive reserve and compensatory adaptive mechanisms in conditions of high intellectual activity.

19.4 Conclusion

Taking into account the high efficiency and innocuous nature of the remedy, one can consider Enoant to be a functional food product that could be used under conditions of high intellectual activity and in complex therapy for stress, e.g. after situations with negative psychological consequences, such as surgical operations, violence, disasters, accidents, etc., under risk of alcohol addiction (e.g. in nations of northern Euroasia and North America who have a genetically pre-determined low tolerance to alcohol). To verify this hypothesis, there is a need to conduct further complex psycho-physiological investigations.

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Chapter 20
Grape Cane as a Source of *Trans*-Resveratrol and *Trans*-Viniferin in the Technology of Biologically Active Compounds and Its Possible Applications

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Abstract Nowadays, the grape cane waste, being the side product of viniculture, still has not found its extensive utilization in the production of medicinal and antipathogenic drugs because of different reasons, such as variability and difficulty in the determination of the chemical composition, sophisticated extraction and high lability of target components. High biological as well as commercial (2,000–3,000 $ per kg) importance of the grape cane stilbenes: *trans*-resveratrol and *trans*-ε-viniferin makes the research in this field highly promising.

20.1 Introduction

The chemical composition of grape cane depends on the *Vitis vinifera* varieties, its vegetation period [1], growing conditions and occurrence of plant diseases, such as downy mildew and oidium [2, 3]. At the same time, the grape cane was shown to be a potential source of biologically active compounds [4]. The major components, which show unique biologically active properties of the grape cane [5–7], are *trans-resveratrol* (3,5,4′-trihydroxystilbene) and *trans*-ε-viniferin. Chemical structure of these compounds could be found in [4, 8].

The content of stilbenes in the grape cane is an indicator of the diseases, which the plant has faced during the year (e.g., mildew, oidium). In the same agricultural climatic conditions of plant growing, the maximal content of stilbenes in the grape cane is found in the more disease-resistant varieties [1].

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20.2 Materials and Methods

In the current work, we have studied 11 varieties of grapes, which were growing in the Sevastopol region (Crimea, Ukraine) during 2009–2011. In February 2011, samples of aqueous ethanolic extracts were prepared from 2 kg of ground product and 4 L of rectified ethanol and were analyzed for carbohydrates, mineral salts and chlorophyll.

Analysis was carried out using a liquid chromatography system (Agilent 1100 series; Agilent Technologies Inc.) equipped with a photodiode array detector (LC-DAD), refractometric detector (LC-RID), autosampler and LC column thermostat.

For the purpose of quantitative determination of stilbenes, the grape cane samples were ground up to 1–2 cm size and extracted with rectified ethanol 3:1 (v/w) for 24 h at room temperature. Then, the samples were placed in an ultrasonic bath for 1 h; after that, the extracts were passed through a 0.45 μm regenerated cellulose membrane filter. Samples of 0.5 μl were injected into a reversed-phase C18 column (Zorbax SB, 1.8 μm, 4.6 mm × 50 mm; Agilent Technologies Inc.) with LC column thermostat at 60 °C. A gradient solvent system was used consisting of 1.3 mM trifluoroacetic acid (solvent A) and methanol (solvent B). The elution profile had the following proportions (v/v) of the solvent B: 0 min, 20 %; 0–2 min, 20–45 %; 2–4 min, 45–100 %; 4–4.5 min, 100 %; 4.6–6 min, 20 %. The solvent flow rate was 1.5 mL min^{-1}. Quantitation was performed using an external calibration curve monitoring peak areas at 306 and 320 nm for *trans*-resveratrol and *trans*-ε-viniferin, respectively.

A carbohydrate chromatographic column (7.8 mm × 300mm, Supelcogel-C610H) was used for the analysis of carbohydrates content. The chromatographic separation of the carbohydrates was achieved by using water with 0.1 % (v/v) phosphoric acid as mobile phase at a flow rate of 0.5 mL min^{-1}. The column temperature was fixed at 30 °C and the injection volume was chosen to be 5 μl. The content of carbohydrates was determined using calibration characteristics for carbohydrate standards with the refractometric detection.

For the general analysis of the dry weight, 10 ml of aqueous ethanolic extracts were placed in porcelain plates and evaporated to dryness at 80 °C in a convection drier, and then the dry residue was weighed. Subsequent heating of these residues in an incinerator at 500 °C gave after weighing the mineral salts content. The chlorophyll content was calculated on the basis of weight difference between total dry content and content of cumulative carbohydrates, stilbenes and mineral salts.

20.3 Results and Discussion

It was determined that the maximal amount of stilbenes can be found in the canes at the end of February – middle of March (Table 20.1). In 2011, the highest content of stilbenes was observed in the following varieties: Riesling, Rkatsiteli, Muscat Hamburg,

Table 20.1 Content of stilbenes (*trans*-resveratrol/*trans*-ε-viniferin) in the grape cane depending on the time of collection (mg/kg dry weight)

Grape varieties	06.08.09	25.03.10	04.07.10	02.08.10	16.11.10	24.02.11
Ranii Vira	65/101	97/724	8/27	15/0	7/219	147/451
Cabernet Sauvignon	22/337	341/615	9/0	30/179	9/180	370/858
Agadai	21/393	524/867	11/17	0/22	8/168	240/619
Aligote	42/564	147/1206	10/0	15/559	9/363	128/704
Merlot	57/236	316/1089	13/0	0/19	9/258	165/335
Rkatsiteli	23/70	46/480	20/0	0/0	9/171	876/865
Riesling	41/896	441/779	34/18	185/296	14/227	1300/1901
Citron Magaracha	16/341	43/468	7/0	43/129	4/130	677/593
Moldova	52/92	62/286	80/29	9/146	21/123	264/419
Muscat Hamburg	16/31	25/713	5/0	17/0	12/369	692/1284
Muscat Golodrigi	40/78	37/246	17/0	21/120	10/153	185/388

Table 20.2 The basic chemical content of grape canes in five grape varieties collected in Sevastopol region, Crimea, Ukraine, in February 2011

Substance content, g/kg dw	Cabernet Sauvignon	Citron Magaracha	Rkatsiteli	Muscat Hamburg	Riesling
Sucrose	5.36	4.17	7.90	7.10	7.09
Glucose	15.34	21.23	19.71	25.71	15.84
Fructose	21.35	27.45	26.48	34.18	21.78
trans-resveratrol	0.37	0.68	0.88	0.69	1.30
trans-ε-viniferin	0.86	0.59	0.87	1.28	1.90
Mineral salts	2.13	2.40	1.12	1.57	1.47
Chlorophyll	18.02	24.23	24.26	31.19	24.00

Citron Magaracha and Cabernet Sauvignon. The content of *trans-resveratrol* and *trans*-ε-viniferin in these varieties was in the range of 0.37–1.30 g/kg and 0.59–1.90 g/kg of dry weight, respectively.

In addition to stilbenes, grape cane contains some additional components (Table 20.2). Carbohydrates, for instance, are of great importance because they can facilitate the dissolution of relatively lipophilic stilbenes into hydrophilic media. As the extraction from the grape cane was performed using an ethanolic rectificate, it is worth mentioning that, in spite of the fact that the concentration of ethanol decreased (up to 78–85 % by volume) due to dilution by the water present in the cane, it was sufficient for incidental chlorophyll extraction. As a consequence of the natural carbohydrates content in the aqueous ethanolic grape cane extracts, it was possible to transfer stilbenes into the aqueous phase during vacuum concentration. Moreover, separation of chlorophyll could be accomplished by simple decantation.

On the basis of control samples of grape cane extracts in eleven grape varieties, 0.5 L of stilbene concentrate was obtained by vacuum concentration to 46.3 % carbohydrates content, as well as 2.9 g/L *trans*-resveratrol and 6.3 g/L of *trans*-ε-viniferin.

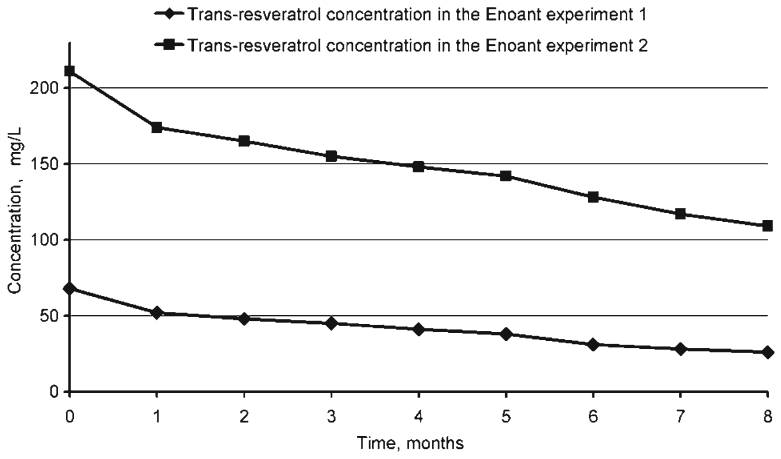

Fig. 20.1 Dynamics of the *trans*-resveratrol concentration in the control samples

Subsequently, control samples from the above-mentioned concentrate were prepared on the basis of the food concentrate Enoant with a starting concentration of *trans*-resveratrol and *trans*-ε-viniferin 68 mg/L, 211 mg/L and 142 mg/L, 485 mg/L respectively (experiment 1 and experiment 2).

The alcohol-free dietary concentrate from the Cabernet Sauvignon grape Enoant has been successfully used for the prevention and treatment of adverse health effects. It contained 18–20 g/L of total polyphenols, which is about ten times more than the polyphenol content of red wines. Various polyphenolic substances were identified in Enoant by HPLC [9]. Enoant contained traces of stilbenes and it was chosen by us as the basis for production of a new dietary product enriched with *trans*-resveratrol and *trans*-ε-viniferin. The primary interest was focused on the stability of stilbenes in the new dietary product.

During 8 months, we examined the dynamics in the decrease of the stilbene concentration in control samples of Enoant, which were kept at room temperature, and in the frozen stilbene extract.

As shown in Figs. 20.1 and 20.2, the highest decrease of stilbene concentration was observed during the first month of storing. After that time, the decrease proceeded at a lower rate. During 8 months, the amount of *trans*-resveratrol and *trans*-ε-viniferin in Enoant decreased to 62 and 49 % respectively (experiment 1), as well as to 48 and 43 % (experiment 2). In case of frozen stilbene concentrate, losses of 6 % of *trans*-resveratrol and 4 % of *trans*-ε-viniferin were observed.

Most probably, the stilbene concentration decreased *via* oxidative degradation. Moreover, interaction with other components of the samples should also be considered [10]. Noteworthy, we found that during both experiments the lower rate of decomposition was observed for Enoant having higher concentration of stilbenes. It should be noted that in the frozen stilbene concentrate, a considerable decrease of

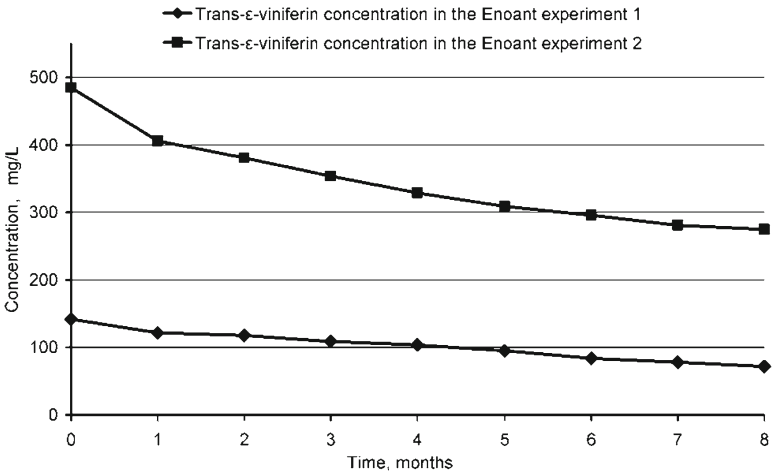

Fig. 20.2 Dynamics of the *trans*-ε-viniferin concentration in the control samples

the stilbene concentration was observed only during the first month, while during the next seven months it was negligible.

Therefore, the storage conditions such as temperature, concentration of stilbenes and substances able to react with stilbenes are crucial factors influencing the storage life of the stilbenes.

20.4 Conclusion

The current work points out at an important issue in the potential production of biologically active products based on the grape canes. We have shown that the high concentration of stilbenes have a direct influence on the storage life of stilbenes. Undoubtedly, the main problem for the practical implementation of this result is the dependence of the stilbene content on the agricultural climatic conditions. As a result, there is a need for a seasonal search for raw materials with the maximal content of stilbenes, which requires complex analytical equipment and accurate sampling for reliable data.

One of the upcoming trends for the application of products containing natural *trans*-resveratrol and *trans*-ε-viniferin is their usage in the production of dietetic food and cosmetic industries basing on their natural antioxidant properties. For the further development of grape cane products, special attention should be paid to the treatment and prophylaxis of oncological and cardiovascular disorders [11].

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Chapter 21
The Effectiveness of Enoant in the Treatment of Bronchitis in Children

Igor Bogadelnikov, Renata E. Verem'eva, and Yuliya Vyaltseva

Abstract We administered Enoant for the first time in 1993 as a part of a complex treatment for children with recurrent bronchitis (RB). These patients typically have significant disorder of their microbial ecological system, enhancement of lipid peroxidation, and decreased immune responsiveness. The reason for choosing Enoant was a high content of flavonoids, its impact on the microbial biocenosis of the intestine due to active acidity, and finally the absence of alcohol in the medication. To study the effect of Enoant on the microbial ecological system of the intestine and the immune system of children with RB, we observed 142 children aged from 4 to 7 years old. All patients were in the state of remission. The nutritional supplement Enoant was administered 7 ml three times per a day before meals, which equals 56 mg of coloring substance per intake. The course of treatment was 15 days. The control groups received the eubiotics Bifikol diet in combination with phytotherapy. Our research has shown the clinical effectiveness of Enoant, which was accompanied by improvement of bacteriological parameters of intestinal microflora and immune indicators in serum, i.e. the increased activity of complement, lysozyme, phagocytic rates of reaction, concentration of immunoglobulins A and M, the number of T-lymphocytes and reduced level of immunoglobulin E and the CEC.

21.1 Introduction

Currently, medical scholars have shown great interest in the ideas of I. Mechnikov who considered that the intestinal microbial homeostasis plays a leading role in determining the state of human health and lifespan, emotional condition, and

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productiveness [1]. From this perspective, the normalization of the intestinal microbial biocenosis is crucial for maintaining human health. A balanced diet is considered to be the most affordable means to influence the eubioz, and, as a result, the condition of the whole body. Functional nutritional products have become a promising solution for the improvement of our health [2, 3]. The products include natural substances, which can be taken daily and which have a regulating effect on physiological functions and biochemical reactions.

We administered Enoant for the first time in 1993 as a part of a complex treatment for children with recurrent bronchitis (RB). These patients' conditions were characterized by a significant disorder of the microbial ecological system, enhancement of lipid peroxidation, and decreased immune responsiveness.

The reason for choosing Enoant was that it had a high content of flavonoids inhibiting lipid peroxidation (later it was found to be 300 times higher than in serum). The antioxidant activity of flavonoids is 10–20 times greater than that of other natural antioxidants (e.g. vitamin E). Polyphenols contained in Enoant are monomeric and oligomeric forms of the molecules, which have strong antioxidant properties. Due to their unique antioxidant properties, polyphenols have anti-inflammatory, antiallergic, antiviral, anticarcinogenic, radioprotective and other effects on the body [4].

Currently, the use of probiotics along with medications that provide adhesion and survival of exogenous microorganisms is regarded as the most promising development in the treatment of intestinal dysbiosis. Enoant provides a unique environment for the colonization of bacteria that form the basis eubiotics. Its impact on the intestinal microbial biocenosis is determined by active acidity, which is equal to pH = 3.5, while the maximal bioactivity of opportunistic bacteria falls within the range of pH = 6.0–8.0, by the decrease in pH down to 4.5 and below resulting in the death of *Shigella*, *Klebsiella*, *Proteus*, and *Candida* fungi, and finally by the absence of alcohol in the medication [5, 6].

Another important consideration was that Dr. Yu. A. Ogay, Enoant's developer, offered it to us free of charge right after the demise of the Soviet Union in 1993. In the wake of the economic collapse, it came very timely.

The purpose of our investigation was to study the effect of Enoant on the microbial ecological system of the intestine and the immune system of children with RB.

21.2 Methods

We observed 142 children with the RB aged 4–7 years old who were treated at Children's Hospital of Simferopol, Ukraine. All patients were in a state of remission. The culture and immunological methods of examination feces, saliva and peripheral blood were used. The results of the study were processed using Statistica 8 software with the calculation of the arithmetic mean (M), the standard error of mean (m) and the level of probability (P). $P < 0.05$ was considered statistically significant.

The bacteriological examination of fecal sampling was performed in a sterile dish within 2 h from the moment of sampling. A sample of 1 g feces was triturated in a sterile mortar with 9 ml of saline.

To isolate pathogenic enterobacteria from this basic dilution (1:10), inoculation of a dense Ploskirev medium (or Levin medium together with sintomitsin) was performed. At the same time, this produced a massive inoculation of native material in enrichment liquid medium such as Muller-magnesium. Then, the main culture was diluted in saline at a concentration of 10^{-3} to 10^{-10}. Finally 0.01 ml of the culture from the last dilution was inoculated on the surface of Endo, Levine, and Saburo media, and into 3–5 % blood agar.

The quantitative content of bifidobacteria was determined in semi-hepatic Blaurokka medium, staphylococci – in yolk-salt agar, *Candida* fungi – in Sabouraud medium with polimeksin, *Enterobacteriaceae* bacteria – in Ploskirev and Levin media, and a 5 % blood agar. The research took into account the percentage ratio between the colonies with and without hemolyzing properties.

The quantitative content of all kinds of microorganisms in 1 g of feces was determined by the number of colonies, which grew in a respective medium, taking into account the volume of inoculum and the degree of dilution.

21.3 Results

In 107 patients with chronic recurrent bronchitis in remission, the main disease manifested itself without obstruction; in 35 cases relapses were complicated with obstruction.

21.3.1 Provision of Colonization of Exogenous Bacteria Included in the Probiotics

The study of quantitative and qualitative composition of feces' microflora revealed the intestinal disbiosis of different degree in 59 % of patients with RB and in 74 % of patients with ROB. Violations of the microbial biocoenosis were characterized mainly by a decrease in number of bifidobacteria, normal *Escherichia coli* and increased level of hemolyzing and enzymely poor *Escherichia*. Thus, in cases with recurrent bronchitis dysbiosis, this was caused mainly by disorders of the aerobic microbiota. As for RB, as well as for ROB, the total number of *E. coli* was reduced by 25 % ($P<0.001$) and 30 % ($P<0.001$) compared to the group of healthy children. Hemolyzing and enzymely poor *Escherichia* were present in 16 and 23 % of patients with RB, respectively, and 19 and 19 % of children with ROB, respectively. Besides the changes of aerobic bacteria, another important manifestation of dysbiosis was the reduction of *B. bifidum*. Thus, 20 % of cases (RB) and 27 % (ROB) exhibited levels of bifidobacteria below 10^8–10^{10}.

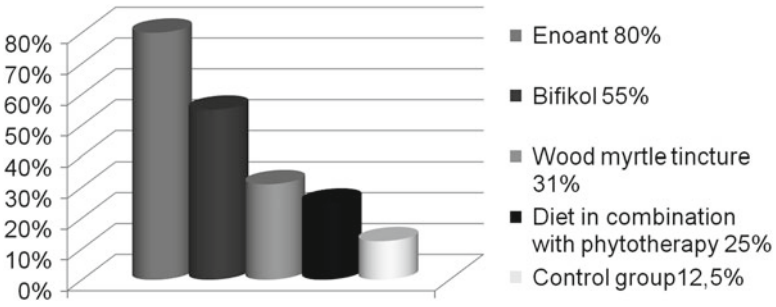

Fig. 21.1 Comparative analysis of various methods effectiveness in treatment of intestinal dysbacteriosis of children with recurrent bronchitis

Increased growth of coccoid forms of microbes was noted, respectively, in 43 and 31 % of patients. The predominance of hemolytic staphylococci among these forms was observed with greater frequency in the ROB, which probably resulted from a lower content of bifidobacteria and normal colon bacillus in microbial structure, with pronounced antagonistic properties.

Due to the discovered disorder of the intestinal microbial biocenosis in children with RB, it was necessary to include in complex therapy the elimination of dysbiosis.

The food supplement Enoant was prescribed at a dose of 7 ml for three times per day before meals at the rate of 56 mg of dye per intake. The treatment lasted 15 days. In the control group, known drugs with similar mechanisms were used, i.e., eubiotics Bifikol, and diet in combination with phytotherapy.

We observed four groups of patients (Fig. 21.1), in which we studied the effect of one of the above described means on the state of microbiota and immunity.

The best results were obtained by including Enoant into the complex therapy. For example, when included in the diet, it causes complete normalization of the microbial structure of 80 % of patients, whereas in the treatment by well-known probiotic Bifikol, normalization of endogenous microflora was observed only in 55 % of cases.

The normalizing effect of Enoant on the microbial biocoenosis results in an increase in the normal *Escherichia coli*, to significantly improve its physiological properties, to help the growth of Bifidobacteria, and to eliminate intestinal fungi *Candida albicans*. This beneficial effect of Enoant on the endogenous microflora is due to the active acidity of Enoant (pH 3.5), whereas, as it is well known, the maximal physiological activity of the majority of opportunistic bacteria is in the range of pH 6.0–8.0. Reducing the acidity to 4.5 leads to the destruction of most of the facultative microorganisms. Also, the bioorganic complex, which is part of Enoant, is an excellent environment conducive to growth, reproduction and improvement of the physiological properties of endogenous microflora.

Polyphenols that are a part of Enoant stimulate the release of gastrin and hydrochloric acid in the stomach and activate the interaction of food proteins with digestive enzymes. This accelerates the transport of proteins through the digestive tract,

Table 21.1 Dynamics of nonspecific immunity markers change in children with RB after the use of Enoant

Parameters	Statistics	Healthy children (n = 22)	With Enoant		Without Enoant	
			Before treatment (n = 20)	After treatment (n = 18)	Before treatment (n = 16)	After treatment (n = 14)
Humoral factors of nonspecific defense						
Complement activity in blood (%)	M ± m $P_1 < 0.05$ $P_2 < 0.001$ $P_3 > 0.05$	51.3 ± 0.9	40.9 ± 1.4	49.4 ± 0.9	40.6 ± 1.4	40.7 ± 0.9
Lysozyme in blood (IU)	M ± m $P_1 < 0.05$ $P_2 < 0.001$ $P_3 > 0.05$	3.7 ± 0.1	2.4 ± 0.1	3.4 ± 0.1	2.7 ± 0.2	2.8 ± 0.1
Properdin in serum	M ± m $P_1 < 0.05$ $P_2 < 0.001$ $P_3 > 0.05$	9.8 ± 0.3	7.7 ± 0.3	9.6 ± 0.3	7.6 ± 0.4	8.1 ± 0.4
Cellular factors of nonspecific defense						
Phagocytic activity	M ± m $P_1 < 0.05$ $P_2 < 0.05$ $P_3 > 0.05$	59.2 ± 1.1	44.6 ± 1.8	51.8 ± 2.8	44.0 ± 1.9	44.9 ± 2.0
Phagocytic rates of reaction	M ± m $P_1 < 0.05$ $P_2 < 0.001$ $P_3 > 0.05$	6.4 ± 0.4	4.3 ± 0.2	5.5 ± 0.2	4.3 ± 0.2	4.6 ± 0.2

Note: *P1* – children with RB vs. healthy children, *P2* – pre- vs. post-treated children received Enoant, *P3* – pre- vs. post-treated children who did not receive Enoant in combination therapy

optimizes their hydrolysis and enhances the absorption of peptides, amino acids, vitamins (including vitamin D), and iron. In this regard, Enoant also can be used to improve appetite, stimulate digestion, prevent rickets, beriberi, and anemia.

21.3.2 Correction of Immune Disorders

The use of Enoant promotes the most important factors of nonspecific resistance and immunological reactivity: lysozyme activity, complement, properdin, the phagocytic activity of neutrophils, increased concentrations of IgA and parameters of T-cell immunity (Table 21.1). Such a beneficial effect on the immune system is mediated through microecological system of the intestine. Microbiocenosis is a major determinant of immunological reactivity. The necessary condition for the proper functioning of the immune system is the sufficient content of endogenous bacteria that perform antagonistic actions, synthesize vitamins, and stimulate the immune system and other functions.

21.4 Conclusion

Our research has shown a high clinical effectiveness of Enoant, which was accompanied by improvement of bacteriological parameters of intestinal microflora and immune indicators in serum, such as increased activity of complement, lysozyme, the concentration of immunoglobulin classes A and M, the number of T-lymphocytes and their functional parameters, reducing the level of immunoglobulin E class and the CEC.

Thus, the above-mentioned biological properties of Enoant (i.e. the antioxidant balance control, normalization of intestinal microflora, absorption and breakdown of nutrients control and the correction of the immune system) make it a unique product of functional nutrition and a perspective solution for the maintenance of health and rehabilitation.

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Chapter 22
Toxicology of Adipose Tissue (Adipotoxicology), or Adipose Tissue as a "Toxicrine" Organ

George N. Chaldakov, Stanislav Yanev, and Victor Georgiev

Abstract In 1994, leptin, adipocyte-derived hormone, was discovered. Onwards, adipose tissue, particularly its "white" phenotype (WAT), has been seen not merely as a lipid store, but as a secretory – endocrine and paracrine – organ, synthesizing, storing, and releasing more than 100 signaling proteins collectively designated adipokines. Human WAT is partitioned into two large depots (subcutaneous and visceral) and many small depots associated with internal organs, e.g. heart, blood vessels, major lymph nodes, pancreas, prostate gland, ovaries, thymus. There is now increasing evidence that exposure to persistent organic pollutants (POPs) may contribute to the pathogenesis of low-grade inflammatory diseases such as atherosclerosis, obesity, type 2 diabetes and metabolic syndrome. Noteworthy, these pollutants accumulate mainly in adipose tissue. Likewise, xenobiotic-metabolizing cytochromes p450 (CYP) are expressed in adipose tissue, where CYP1A1 and CYP1B1 can bioactivate xenoestrogens (endocrine disruption xenobiotics) and carcinogenic polycyclic aromatic hydrocarbons. The present article highlights (i) the secretion in adipose tissue and its dysfunction related to the pathogenesis of various diseases, and (ii) the storage and metabolism of POPs, a phenomenon herein referred to as "toxicrine" activity of adipose tissue. Such a research process may frame a novel field of study, adipotoxicology.

> The adipose tissue in the human body is there for the best, the bad, and the worse!
> Paraphrase from Charles Lapiere and Erik Maquoi [1]

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22.1 Introduction

The Big Bang model is the prevailing cosmologic hypothesis of the early development of the universe, suggesting that it was once in an extremely hot and dense state that expanded rapidly about 13.7 billion years ago. The Brain's Big Bang was described by Gerald M. Edelman and Giulio Tononi in their book *A Universe of Consciousness: How Matter Becomes Imagination* (New York: Basic Books 2000). Accordingly, the Fat's Big Bang has explored on 1 December 1994, the time of the first publication about leptin, an adipocyte-secreted hormone (Jeffrey Friedman et al., *Nature* 1994, 372:425–432), followed by an expanded research in adipobiology and adipopharmacology of disease.

Recently, obesity and related cardiometabolic diseases, such as atherosclerosis, hypertension, type 2 diabetes and the metabolic syndrome, are among the major physical, social and economic burdens, globally. The World Health Organization has predicted a "globesity epidemic" with more than one billion adults being overweight (BMI over 25 kg/m^2) and at least 400 million of these being clinically obese (BMI over 30 kg/m^2). The metabolic syndrome affects about 25 % of adults in the United States, and its prevalence continues to increase as obesity and lack of physical activity become progressively more common (not only) in modern society. Arguably, we have learned more about the molecular control of food intake and energy homeostasis, particularly, the role played by adipose tissue in the pathogenesis of cardiometabolic, liver, malignant and neurodegenerative diseases.

22.2 Adipose Tissue

There are two main types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). Each of them is a dynamic multicellular and multifunctional assembly composed of adipocytes (fat cells) and non-fat cells. In WAT, these later cell types include stromal-vascular cells (fibroblasts and endothelial, immune and stem cells). At birth, the average-size infant has approximately 5 billion adipocytes, whereas – approximately 80 billion in adult.

Adding to them billions of stromal-vascular cells, makes the whole body WAT a major human's secretory organ. **A**dipose tissue (hereafter to be considered WAT) is partitioned into two large depots (subcutaneous and visceral) and many small depots associated with heart, blood vessels, major lymph nodes, pancreas, prostate gland, ovaries, thymus (Fig. 22.1). Accordingly, two major subfields of adipobiology have emerged, adipoendocrinology (studying the endocrine activity of adipose tissue) and adipoparacrinology (studying the paracrine activity of adipose tissue); both dealing with the pathogenesis of obesity-related diseases [2–21].

Conventional association of the word "adipocyte" usually refers to the lipid-filled cell found in various body locations. However, it is much more than that. In 1987, adipsin, a circulating serine protease of complement system, was found as the first discovered endocrine product of adipocytes. However, it was since 1994

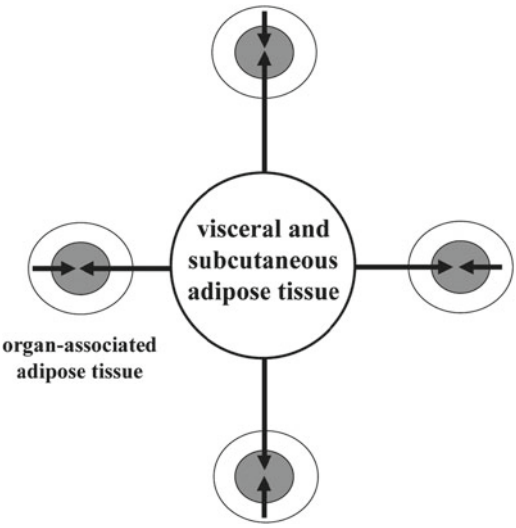

Fig. 22.1 Schematic illustration of a large adipose depot (visceral and subcutaneous adipose tissue) and small adipose depots (organ-associated adipose tissue). Endocrine action (*long arrows*) and paracrine action (*short arrows*) of adipokines and of other adipose-derived signals on various organs. Organ parenchyma (*grey*) is surrounded by adipose tissue (*white*)

Table 22.1 Characteristics of Leptin

Molecular weight	16 kDa
"Birth place"	Jeffrey Friedman's Laboratory of Molecular Genetics, The Rockefeller University, New York City, NY, USA
Gene	*ob*(*Lep*) gene located on chromosome 7 in humans
Receptors	6 Ob-R isoforms (Ob-Ra – Ob-Rf), *db* gene splicing products; 1 sOb-R, ADAM10 shedding product
Functions	Food intake/energy balance, cell growth, reproduction, immunity/inflammation, thrombogenesis, angiogenesis, osteogenesis, neuroprotection, developmental programming, surfactant expression by alveolar type II pneumocytes
Sources	Adipocytes, gastric epithelial cells, ovaries, bone marrow, placenta, mast cells, liver, brain, lung alveolar interstitial fibroblasts (lipofibroblasts)

when leptin, a circulating cytokine (Table 22.1), was discovered which has been triggering further studies on the secretory potential of adipose tissue. Recent genomics, proteomics and microarray methodologies have dramatically increased the number of adipose tissue-secreted molecules, conceptually named adipokines. Adipose tissue's secretome includes adipokines (cell growth factors, cytokines, chemokines, neurotrophic factors, neuropeptides, and hypothalamic hormones/releasing factors), steroid hormones, all components of renin-angiotensin system, free fatty acids, fatty acid binding protein-4 (FABP-4), prostaglandins, endocannabinoinds, also nitric oxide (NO), hydrogen sulfide (H_2S) and homocysteine. As salutogenic (health protective) adipokines we may list: adiponectin, leptin, interleukin-10 (IL-10), IL-1 receptor agonist (IL-1Ra), nerve growth factor (NGF), brain- derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), vascular endothelial growth Factor (VEGF), neuropeptides, metalothioneins 1–3, C1q/TNF-related protein-3 (CTRP-3), a LPS antagonist, and sirtuins

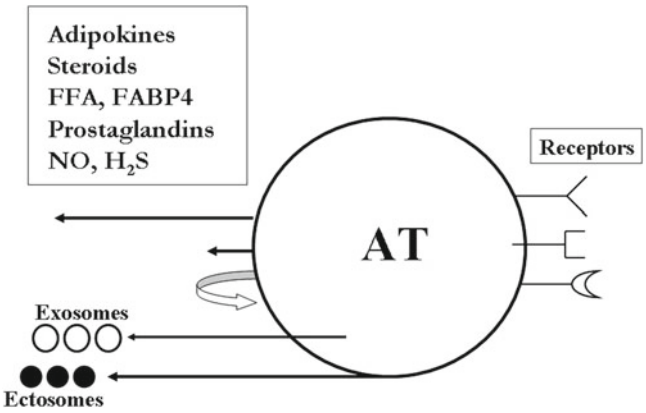

Fig. 22.2 Schematic illustration of adipose tissue as a secretory (endo-, para- and autocrine) and responsive organ

Table 22.2 Thomas Kuhn's paradigm shift in adipobiology

From	To
Adipose tissue is a lipid/energy storage organ implicated in obesity	Adipose tissue is an endocrine and paracrine organ
	Adipose tissue is an immune and inflammatory organ
	Adipose tissue is a steroidogenic organ
	Adipose tissue is a "juxstaglomerular organ"
	Adipose tissue communicates with brain (neuroadipology)
	Adipose tissue stores and metabolizes toxic xenobiotics (adipotoxicology)
	Adipose tissue is thus implicated in numerous diseases beyond obesity

(SIRT1, SIRT3). In effect, adipose tissue is a multifunctional – secretory and responsive – organ (Fig. 22.2). Cardiometabolic and other obesity-related diseases are characterized by a chronic, low-grade inflammatory phenotype of the adipose tissue. This inflammatory phenotype is most likely initiated by dysfunction of adipose secretion and fuelled by infiltrating macrophages, lymphocytes and mast cells [22–34]. Altogether, these and other excellent studies have shifted the paradigm of adipose tissue from simple energy storage to the body's major endocrine and paracrine organ (Table 22.2).

22.3 Toxicology of Adipose Tissue (Adipotoxicology)

Even in a lean person, adipose tissue represents about 15–20 % of body weight [35], including external (subcutaneous and visceral) and internal (organ-associated) adipose tissue (Table 22.3). In *Homo obesus* this percentage can increase by more than

Table 22.3 Adipotopography (fat mapping): variations

TOFI**	Thin outside, fat inside
TOTI*****	Thin outside, thin inside
FOFI*	Fat outside, fat inside
FOTI**	Fat outside, thin inside

Note: The number of asterisks indicates the quality of cardiometabolic health, as related to adipose tissue distribution. Thus, TOTI represents a highest quality and FOFI – the lowest [49]

50 %. Since about 70 % of adipose tissue mass is composed of lipids, the adipose tissue represents a major reservoir for many different lipophilic xenobiotics; here, they may modulate the activity of key transcription factors engaged in the control of differentiation and secretion of adipose tissue, and thus implicate in the pathogenesis of cardiometabolic diseases [36–45].

Since most of these lipophilic xenobiotics are resistant to biological and chemical degradation, they were dubbed persistent organic pollutants (POPs). There is now increasing evidence that exposure to POPs including chlorinated pesticides and polychlorinated biphenyls may contribute to the pathogenesis of low-grade inflammatory diseases such as atherosclerosis, obesity, type 2 diabetes and metabolic syndrome. Likewise, xenobiotic-metabolizing cytochromes p450 (CYP) are expressed in adipose tissue, and are inducible through mechanisms similar to those in the liver. Accordingly, CYP1A1 and CYP1B1 can bioactivate carcinogenic polycyclic aromatic hydrocarbons (benzo(a)pyrenes) and xenoestrogens (bisphenol A, phthalates, dioxin, and organochlorine pesticides). Altogether, an adipocentric approach underlines the need for human biomonitoring of POPs accumulation and metabolism, as related to the pathogenesis of disease.

There are several pathways known in cell secretion – endocrine, paracrine, autocrine, and intracrine, also exosomes and ectosomes (reviewed in [7]). Here we introduce a "toxicrine" pathway, meaning that adipose tissue is able to store, metabolize, and release toxic xenobiotics. Overall this research process may frame a novel field of study, adipotoxicology. An intriguing topic in the field may be the evaluations of possible effects of adipose-stored xenobiotics over the secretion of adipokines [46, 47] and/or other adipose-derived bioactive compounds.

On important conclusion from already known data is that although the effects of environmental obesogens of early-life exposure are irreversible such people can reduce later harmful health effects by healthier lifestyle including minimizing/excluding the usage of plastics and other POPs in their life. As Dr. Thayer stressed in the recent Workshop dedicated to the problems of "Environmental Chemicals in Diabetes and Obesity": "We were surprised at the number of chemicals that seem to be interacting with signaling pathways involved in weight regulation." She added that evidence also suggests these same bioactive compounds are linked with diabetes and metabolic syndrome, an understudied but natural research direction that brings together the obesity and diabetes issues [48].

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Chapter 23
Opportunity of Remediation of Radionuclide-Contaminated Soils and Growing Ecologically Pure Plant Material via Water-Retaining Polymer

Anna Tadevosyan, Michael Schellenberg, Stepan Mayrapetyan, and Laura Ghalachyan

Abstract One of the important aspects for remediation of contaminated soils is a reduction of biological mobility of radionuclides in the water-soil-plant system. Anthropogenic radionuclides ^{137}Cs and ^{90}Sr become more concentrated as they move up the food chain often becoming human health hazards. The aim of the research is to elucidate the influence of polymers' impact on biological migration of ^{90}Sr and ^{137}Cs in the system irrigation water-soil-plant in zones of radioecological tension. The tests were carried out in soil without and with application of polymer Ca^{++} in root-inhabited media (RIM) in the most radioecological tension zone of Armenia. Sweet basil (*Ocimum basilicum* L.) was chosen for the investigations. Both quantitative and qualitative productivity of basil depending on (1) the presence or absence of Ca^{++} polymer, (2) quantity of Ca^{++} polymer, (3) content of artificial radionuclides in different soil layers and (4) type of plant tissue were determined. The presence of Ca^{++} polymer in soil RIM promoted the decrease of ^{90}Sr content in basil leaves 1.7–2.0 times compared to the control sample. The use of 1 g/plant Ca^{++} polymer decreased the content of artificial radionuclides in the soil layer at the end of vegetative period: 1.5 times for ^{90}Sr and 1.3 times for ^{137}Cs. Ca^{++} polymer was effective for decreasing ^{90}Sr concentration in sweet basil.

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23.1 Introduction

Ecosystems throughout the world have been contaminated with radionuclides (RN) by aboveground nuclear testing, nuclear reactor accidents and nuclear power generation [1–3]. Even normalized emissions of atomic industry entities and nuclear power engineering facilities gradually, in the course of time, accumulate in soils and then enter the agricultural production chain. Soil contaminated with RN poses a long-term radiation hazard to human health through exposure mainly via the food chain. Therefore, constant supervision and remediation of contaminated soils is an urgent need for any country throughout the world.

Radioisotopes characteristic of nuclear fission are released into the environment and become more concentrated as they move up the food chain often becoming human health hazards. Amongst the man-made RN, ^{137}Cs and ^{90}Sr exert long-term after-effects of radionuclide-derived contamination, as the half-lifes are 30.1 and 28.6 years for ^{137}Cs and ^{90}Sr, respectively. A large portion of some RN, including ^{137}Cs, would remain in the root-zone soil for many years after their deposition. This means that a radioactive deposition onto soil can lead to a root uptake lasting for many consecutive years [4].

The ability to predict the consequences of an accidental release of RN relies mainly on the level of understanding of the mechanisms involved in radionuclide interactions with different components of agricultural and natural ecosystems [5]. One of the important aspects of the contaminated soils remediation is an understanding of protective actions aimed at the reduction of biological migration of RN in a water-soil-plant system. The agricultural radioecology has acquired significant experimental information on the methods for decreasing the transfer of RN from soils to plants. A number of agrochemical and land treatment techniques are available: application of mineral and organic fertilizers, clay materials, liming of soils, changes to the irrigation regimen, and burying of the upper layers of the soil in lower layers [6, 7].

Soils possess high adsorptive capacity towards RN and are the initial point of entry for RN migration into the agricultural production chain. The passage of RN, in particular ^{137}Cs and ^{90}Sr, occurs from the soil solution and the irrigation of water through the root system of plants [4, 8, 9]. Ionic absorption has a specific role in this process of radionuclide transition from the soil into a plant. Radioactive cesium absorbance significantly decreases with the increase of the potassium ion concentration in the soil. This has been confirmed by research performed in Russia and Belarus after the accident at the Chernobyl Nuclear Power Plant [9, 10]. It was discovered that a highly effective means to avert the increasing concentration of exposure to ^{137}Cs for cultivation of cereals can be expected from an optimization of mobile potassium contents in sod-podzolic loamy sandsoils with density of ^{137}Cs contamination more than 925 kBq/m^2 [11]. For ^{90}Sr contamination it could be achieved by liming of sod-podsolic loamy sandsoils with contamination density of ^{90}Sr more than 12 kBq/m^2 [12].

Numerous studies have shown that ^{137}Cs and ^{90}Sr are not removed from the top 0.4 m of soil even under high rainfall. The migration rate from the top few

centimeters of the soil is slow [13, 14]. The top 0.4 m of soil is the region where plant roots are actively accumulating large amounts of elements. Removal of these RN from contaminated soils by plants would provide a reliable and economical method of remediation [15–18].

Currently, the application of potash fertilizers is the main agrochemical treatment utilized to limit the passage of ^{137}Cs from the soil into the plants [9, 19]. The elevation of the concentration of Ca^{2+} ions in the soil facilitates the decrease of ^{90}Sr accumulation by plants due to an increased "concurrence" of these ions [20]. Although the ions Ca, K and NH_4 are characterized by chemical similarity, they do not behave like absolute analogues in the soil-plant system. When K^+ is added to the soil, the content of ^{137}Cs barely decreases, and the addition of NH_4 significantly increases the specific activity of the radionuclide [21].

The water-expanding polymer additives for soil application are highly promising for improving the effectiveness of irrigation-based agriculture. Modern superabsorbent polymers (SAPs) are made from partially neutralized, lightly cross-linked polyacrylic acid. The neutralized polymer is capable of absorbing the surrounding water many times its own weight. Alkaline salts of polymers may be selected from sodium, potassium, lithium and ammonium. The difference in the water-retention ability of polymers with different counter ions allows one to speculate that application of water retaining polymers can influence the transport of RN. SAPs are materials that have the ability to absorb and retain large volumes of water and aqueous solutions. The problem of creation and study of structure and properties of highly swelling hydrogels is the issue of the day and is still getting more urgent due to significant expansion of spheres of its application and usage.

Our preliminary studies have demonstrated that upon application of Supersorbent-87 for plant production there was an observed increase of ^{137}Cs and ^{90}Sr content in plant biomass [22]. It was observed that application of Ca^{2+}-based polymer decreased the content of ^{90}Sr in pepper fruit 1.2 times compared with control sample (without polymer), but did not influence the content of ^{137}Cs [23].

The objective of this research is to elucidate the influence of the quantity of SAP on the yield and radiochemical characteristics of basil, as well as biological migration of ^{90}Sr and ^{137}Cs in the irrigation water-soil-plant system and in zones of radioecological tension.

23.2 Materials and Methods

Tests were carried out in soil without and with an application of polymer Ca^{++} in RIM in the radioecologically tension zone (within a 7 km radius of the Armenian Nuclear Power Plant (ANPP), village Taronik, the Ararat Valley). Basil (*Ocimum basilicum* L.) historically cultivated in Taronik was the crop chosen for this investigation.

Different quantities of Ca^{++} polymer were tested. Ca^{++} polymer was synthesized in the Institute "Plastpolymer", Yerevan, Armenia.

Basil was planted at a density 20 plants/m^2. The experiments were carried out with the following samples: (1) Control-without polymer, irrigating frequency (IF) once 2–3 days; (2) Soil (RIM) + polymer Ca^{++} (1 g/plant), IF once in 3–4 days; (3) Soil (RIM) + Ca++ polymer (1.5 g/plant), IF once in 3–4 days.

The content of RN was determined by radiochemical methods using UMF-1500 [24]. The content of essential oil in dry leaves was determined by a steam distillation method [25]. The water concentration in the leaves was determined by the refractometry [26]. Soil samples for radiochemical analysis were taken from the experimental field before the planting of basil seeds and after crop harvest.

A statistical analysis of collected data was carried out using GraphPad5 software.

23.3 Results and Discussion

The content of ^{90}Sr and ^{137}Cs was determined for the different soil-layers (0–10 CM; 10–20 cm) (Table 23.1). It was found out that in 0–10 cm content of ^{90}Sr and ^{137}Cs exceeded 10–20 cm soil-layer by about 1.3 times.

The indices of basil productivity are provided in Table 23.2. The use of the polymer together with decrease of IF provided an opportunity to obtain the same yield of basil fresh leaves compared with control sample. However, the control plants were notable for their fresh mass of roots, which exceeded polymer-treated samples by about 1.2 times, irrespective of polymer quantity.

It must be mentioned that the use of polymer had no impact on the leaf to stem ratio for above-ground biomass (Fig. 23.1).

Examination of the leaf water showed (Table 23.3) that the use of polymer under the soil did not influence total water content in basil leaves, but the bound water content and the cell sap osmotic pressure decreased by about 10 %.

Table 23.1 Content of RN in different soil-layers in the vicinity of the ANPP

		Content of RN, Bq/kg	
Sample taking time	Depth of soil layer, cm	^{90}Sr	^{137}Cs
May, until the planting of seedlings	0–10	11.5 ± 0.7	12.3 ± 0.2
	10–20	9.1 ± 0.1	9.5 ± 0.6

Table 23.2 Indices of basil productiviy (fresh mass) depending on the presence and quantity of polymer Ca^{++}, g/plant

Samples	Above-ground part	Leaves	Stems	Roots
1	289	188[a]	101	11.7
2	326	214[a]	112	9.9
3	308	197[a]	111	9.5

[a]Tukey's multiple comparison test ($P < 0.05$)

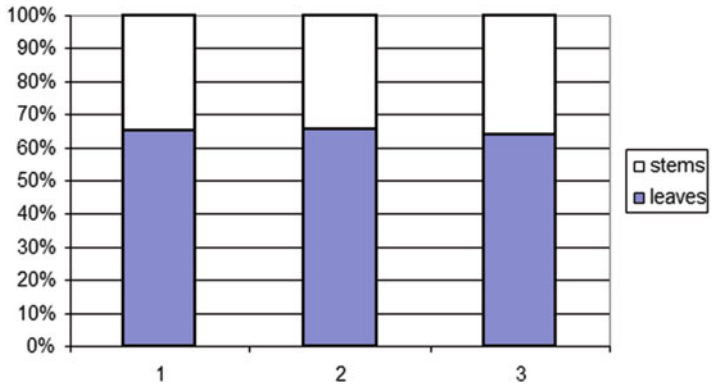

Fig. 23.1 Ratio of leaves to stems in the above-ground biomass of basil. *1* – Control (without polymer); *2* – Soil (RIM) + polymer Ca^{++} (1 g/plant); *3* – Soil (RIM) + polymer Ca^{++} (1.5 g/plant)

Table 23.3 Influence of different quantities of polymer Ca^{++} addition in the RIM on the physiological indices of basil (August)

	Samples		
Indices	1	2	3
Total water content, %	87.6	87.5	88.1
Free water content, %	54.5	57.0	58.4
Bound water content, %	33.1	30.5	29.7
Free and bound water ratio	1.6	1.9	2.0
Osmotic pressure of cellular fluid, atm.	4.23	3.91	3.84
Osmotic bound water, %	11.0	12.4	10.1
Colloid bound water, %	22.1	18.1	19.6

Table 23.4 Influence of different quantities of polymer Ca^{++} addition in the RIM on the content of essential oil in dry basil leaves

Samples	Content of essential oil, %
1	0.71 ± 0.02
2	0.80 ± 0.04
3	0.77 ± 0.2

The content of essential oil in basil leaves is provided in Table 23.4. The data showed that, in the case of using polymer, the content of essential oil in basil dry leaves increased by 8–13 %. The quantity of the polymer had no significant impact on the amount of the essential oil biosynthesis.

The ^{90}Sr and ^{137}Cs contents at the end of vegetation period in different soil layers are presented in Table 23.5. In the control sample, ^{90}Sr content in 0–10 cm and 10–20 cm soil layer was about 60 and 40 % of its original content, and, in of using 1 and 1.5 g/plant Ca^{++} polymer, it was about 55 and 45 %, respectively (Table 23.5).

Table 23.5 Content of artificial RN in the soils in the vicinity of the ANPP (cover crop-basil)

Sample taking time	Samples	Depth of soil layer, cm	Content of RN, Bq/kg	
			^{90}Sr	^{137}Cs
October, after crop harvest	1	0–10	12.8[a]	10.3[a]
		10–20	8.4[a]	5.8[a]
	2	0–10	7.3[a]	6.8[a]
		10–20	5.9[a]	4.9[a]
	3	0–10	8.0[a]	11.9[a]
		10–20	6.4[a]	7.9[a]

[a]Tukey's multiple comparison test ($P<0.05$)

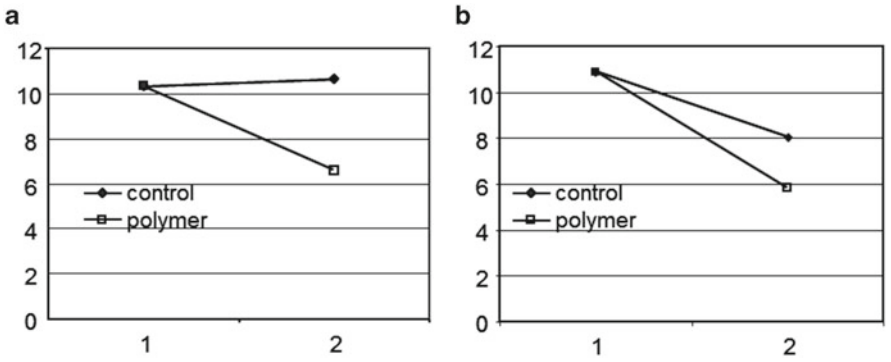

Fig. 23.2 The content of ^{90}Sr (a) and ^{137}Cs (b) in the 0–20 cm soil layer (cover-crop basil), Bq/kg; *1* – before planting, *2* – after harvest

For ^{137}Cs, the concentrations were: control sample 64 and 36 %, for polymer Ca^{++} samples – 58–60 and 40–42 %. It was observed that, for the control sample, the content of ^{90}Sr and ^{137}Cs in 0–10 cm soil-layer exceeded the 10–20 cm depth by 1.5 and 1.8 times, respectively. However, in the polymer samples, ^{90}Sr content in 0–10 cm soil-layer exceeded the one in 10–20 cm soil-layer by 1.2 times and ^{137}Cs content – by 1.4–1.5 times. The data shows that ^{90}Sr and ^{137}Cs water migration from upper 0–10 cm to down 10–20 soil layers in the polymer samples was more intensive than in the control.

In case of 1 g/plant polymer Ca^{++} applied with RIM after basil harvest (Fig. 23.2), we observed that the content of ^{90}Sr decreased in 0–20 soil layer by 1.6 times and the content of ^{137}Cs – by 1.9 times (in comparison with the data shown in Table 23.1). In the control samples, there was no change of ^{90}Sr content, but ^{137}Cs content decreased by 1.3 times. For all samples, the migration of ^{90}Sr was greater than for ^{137}Cs.

The results of the radiochemical analysis of different parts of basil are provided in Table 23.6. For the control samples, the content of ^{90}Sr exceeded the Ca^{++} polymer-treated samples by 2.0 and 1.6 times in basil leaves and by 1.5 and 2.0 times in stems, respectively. In roots, a significant difference of ^{90}Sr content was observed

Table 23.6 Content of artificial RN in different organs of basil, Bq/kg

Organs	Samples	^{90}Sr	^{137}Cs
Leaves	1	11.0[a]	10.9[a]
	2	5.4[a]	14.6[a]
	3	6.6[a]	13.2[a]
Stems	1	3.6[a]	7.4[a]
	2	2.4[a]	15.4[a]
	3	1.8[a]	14.1[a]
Roots	1	3.0[a]	4.2[a]
	2	2.7[a]	8.1[a]
	3	2.4[a]	9.1[a]

[a]Tukey's multiple comparison test ($P<0.05$)

Table 23.7 Observed Ratios of ^{137}Cs-^{90}Sr pair of basil in the system soil-plant

	In soil-plant chain		In biological chain		
Samples	Soil-leaf	Soil-stem	Soil-root	Stem-leaf	Root-stem
1	1.4	2.7	2.0	0.5	1.3
2	3.0	7.1	3.3	0.4	2.1
3	1.4	5.6	2.7	0.2	2.0

only between the control and 1.5 g/plant Ca^{++}polymer-treated samples. In the roots, the accumulation of ^{90}Sr was 25 % higher for the control samples.

Noteworthy, ^{90}Sr content, for the control samples, was greater than leaves and stems of the polymer-treated samples. Apparently, in the polymer-treated samples, the presence of Ca^{2+} in RIM of plant promoted Ca absorption by the plant roots from soil, as Ca is a chemical analog of ^{90}Sr. Besides, Ca^{2+} prevented the migration of ^{90}Sr in root-stem and stem-leaf biological chains of the plants.

The lowest content of ^{137}Cs was observed in the control sample plants, which was less than in the 1 and 1.5 g Ca^{++} polymer-treated samples in leaves by 1.2 and 1.3 times, and in the stems and roots by about 1.9–2.2 times. In the different tissues of basil, the content of ^{137}Cs exceeded ^{90}Sr (with the exception of the control leaves, where ^{137}Cs and ^{90}Sr have the same value) in the leaves of the polymer-treated samples by 2.0–2.7 times, in the control stems by 2.0 times, in the stems of the polymer-treated samples by 6.4–7.8 times, for the control roots by 1.4 times, and in the roots of the polymer-treated samples by 3.0–3.8 times.

The data (Table 23.7) showed that, for all samples, the observed ratio (OR = ^{137}Cs/^{90}Sr in plant:^{137}Cs/^{90}Sr in soil) is >1. This confirms that the absorption of ^{137}Cs from soil by basil is more intensive than of ^{90}Sr with the following trend stems > roots > leaves. This is also confirmed by the data in Table 23.8, according to which the ^{137}Cs accumulation coefficient (AC = RN content in plant:RN content in soil) in the leaves, stems, and roots of basil is greater than AC of ^{90}Sr for all samples. The opposite trend is observed in the stem-leaf chain of basil, i.e., OR of ^{137}Cs-^{90}Sr pair is <1, and the migration of ^{90}Sr, which exceeded ^{137}Cs and in root-stem chain, greatly exceeded the migration of ^{137}Cs in the polymer-treated samples.

Table 23.8 Accumulation coefficients of RN in different organs of basil

Samples	AC	Leaf	Stem	Root
1	^{90}Sr	1.0	0.4	0.3
	^{137}Cs	1.4	0.9	0.5
2	^{90}Sr	0.8	0.4	0.4
	^{137}Cs	2.5	2.6	1.4
3	^{90}Sr	0.9	0.2	0.3
	^{137}Cs	1.3	1.4	0.9

23.4 Conclusion

- The evidence of water retaining polymer in soil RIM demonstrates an opportunity to decrease the consumption of irrigating water as a result of its ability to decrease the quantity of ^{137}Cs and ^{90}Sr contained in water.
- The presence of the polymer Ca^{++} (1 and 1.5 g/plant) in RIM of soil promoted the decrease of ^{90}Sr content compared to the control sample: in basil leaves – by 2.0 and 1.7 times, in basil stems – by 1.4 and 1.8 times and in basil roots – by 1.1 and 1.2 times. At the same time, for the polymer-treated samples, the increase of ^{137}Cs content was observed: in leaves – by 1.3 and 1.2 times, in stems – by 2.1 and 1.9 times and in roots – by 1.9 and 2.2 times.
- The presence of 1 g/plant polymer Ca^{++} decreased artificial RN content in the soil layer (0–20 cm, cover-crop basil) at the end of vegetation period: by 1.4 times for ^{137}Cs and by 1.6 times for ^{90}Sr in comparison with no polymer present.
- Increasing the polymer quantity had no influence on the yield of basil, so for both the productivity of the plants and the biomass radioecological point of view, the use of 1 g polymer Ca^{++} is appropriate.

Acknowledgments The research has been funded under A-1671 ISTC.

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Chapter 24
Bioactive Compounds of Crimean Wines Countering the Stress Experienced by Personnel

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Abstract As was established in previous investigations, consumption of grape's polyphenols, containing in food concentrate Enoant, increase effectiveness of recuperative sanitaria treatment in patients with arterial hypertension and chronic bronchitis. In current article, we present the results of investigation of the wine stress-limiting effects caused by alcohol and polyphenol components. The investigation was performed in 60 patients with arterial hypertension, 60 patients with chronic bronchitis and 60 patients with chronic fatigue syndrome. White table dry wine was used under complex recuperative sanitaria treatment in patients with arterial hypertension and chronic bronchitis and the Ukrainian sparkling wine was used under complex recuperative spa treatment in patients with chronic fatigue syndrome. The dry wine was prepared from white grape "Rkacitely" along with Kakhetian technology. The analysis of data reveals that the most positive wine effect is a successful coping with stress. We had seen significantly increased changes in psychological and morphological reactions, including positive dynamics of the following: patient's self indicated feeling, amount of total complaints and stress reactions, adaptation, diastolic blood pressure, minute blood volume, right carotid artery rheographic index and Gentch test. Also, about quarter of patients had noted the diminishing of wish to drink strong alcohol beverages. The results are in good accordance with the previously known action of grape's wines to prevent heart and vessels diseases.

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24.1 Introduction

The bio-antioxidants had taken an appreciation as an important vital resource for human organism. Biochemical mechanism of the bio-antioxidant action is change of balance between the reactive oxygen substances (ROS) generation and enzyme oxidation towards the last. It makes an optimal condition for metabolism, cell and tissue growth and prevention of various diseases. The bio-antioxidant deficiency usually diminishes tolerance to factors activating the ROS generation, such as stress, ionizing radiation, trauma and pollution. There is a functional antioxidant system (AS) in human organism consisting of enzyme component (including cytochrome P-450) and non-enzyme component (including polyphenols). The ROS generation to AS activity ratio indicates antioxidant status of the human organism.

It is already known that grape polyphenols have reliable curative and preventive effects consisting of reduced oxidation of the low density lipids, decreased platelet aggregation, slower development of increased tonus of arterial smooth muscles, induced coronary blood flow, diminished frequency of heart attacks and myocardial infarction [1–5].

Polyphenols are bound by proline of collagen and elastin proteins found in arteries, which improves their resistance to blood pressure and restores normal NO synthesis in the epithelia, and this synthesis, in turn, regulates vascular relaxation [6, 7].

Very small amounts of polyphenols are assimilated by the human organism via direct consumption of grape berries. When subjected to modern processing technologies, they can be made biologically available and administered at substantially higher doses as a part of grape wines or nutritional (dietary) concentrates, such as Enoant. The alcohol-free dietary concentrate Enoant contains 18–20 g/l of total polyphenols compounds (PC) of Cabernet Sauvignon variety, including 3–5 g/l of coloring substances [8].

Clinical effects of Enoant were studied during complex treatment of patients with stress-induced diseases, such as arterial hypertension (AH), chronic cardiac ischemia (CCI) and chronic bronchitis (CB). Many effects of PC are common both for chronic bronchitis and other diseases, such as activation of the antioxidant system of the organism and reduction of arterial pressure. The PC had pronounced positive effects on patients with AH in relation to parameters of the cardio-respiratory system and other systems of the organism, including improvement of the auscultative respiration pattern, increase in respiratory volume, decreases in minute blood volume and heart capacity, a reduction in heart rate frequency and respiration rate, a decrease in β-lipoprotein content and an increase in blood serum catalase activity, and also an increase in the color index. The specific benefits from administration of PC in patients with CB are reduction in labored respiration and cough, an increased value of the Hench test, an increased erythrocyte amount in parallel with increased red blood cells capacity, a decreased number of leucocytes and reduced stress level showed by the Garkavi test, a normalized minute respiratory volume, a reduction in heart rate frequency, an increase in maximal volumetric expiratory flow rate at a level of 75 and 50 % of forced vital volume, and a lower indexes of Robinson and Kerdo.

Complex therapy received by Enoant-treated patients with AH, CCI and CB has entailed a desire to reduce consumption of alcohol beverages, which are often accompanied the stress. By the end of the course, a reduction in willingness to consume wine registered in 57 % of patients with AH, in 37 % of patients with CCI and in 16 % of patients with CB [9–11].

The above shows that effects of PC are related to successful achievement of a number of clinical goals, such as (1) reduction in action of risk factors, (2) reduction in stress and elimination of distress, (3) functional correction of vegetative nervous system, (4) reduction in intensity of inflammatory processes, (5) improvement in evacuation function of the bronchi, (6) normalization of external respiration; (7) normalization of parameters of blood oxygen-transport function, (8) normalization of arterial pressure and blood circulation, (9) improvement in effectiveness of cardio-respiratory system and increase in its functional reserves; (10) normalization of lipid exchange; (11) increase in detoxification reserves and antioxidant potential; (12) increase in tolerance to physical exercise, (13) enhancement of feeling of well-being as a patient own integral evaluation of the current status [9–11].

Alcohol beverages have well known stress-limiting action [1–5, 12–14]. Thus, wine has to be a good functional food for patients suffering from stress-related diseases, such as AH, CB, and chronic fatigue syndrome (CFS). However, alcohol has negative effects too. For example, usual daily consumption of ethanol (more than 25 ml in man and 15 ml in woman) leads to high risk of AH.

The main goal of the present study was to prove the ability of grape wine, rich in PC, to counter the stress in patients with AH, CB and CFS.

24.2 Materials and Methods

Clinical trial of white table dry wine (DW) was performed in the groups of 60 patients with AH and 60 patients with CB, and clinical trial of Ukrainian sparkling wine (SW) was performed in the group of 60 patients with CFS. All patients were treated at spa sanitaria in Yalta, Ukraine. CFS is the psycho-somatic disease caused by chronic stress and distress. The CFS was diagnosed in accordance with modern criteria [15]. The clinical trials were performed accordingly to GLP and GSP standards. Every patient had standard complex recuperative treatment. The patients were divided into two groups: (a) with wine consumption in addition to standard treatment, and (b) without wine consumption, with standard treatment alone.

The white table dry wine (DW) was prepared from “Rkacitely” grape variety using Kakhetian technology. The technology allows increasing PC content in wine. There are alcohol (12.7 %), organic acids (4.5 g/l) and PC (1700 mg/l), including procyanide (780 mg/l) in DW, whereas saccharine is absent. Dose of DW was 250 ml ones a day, total amount of consumed DW was 3,133 ± 463) ml for 12.5 ± 1.9 drinks. There are alcohol (11.3 %), saccharine (40 g/l), organic acids (5.9 g/l) and PC (162 mg/l), including traces of procyanide, in Ukrainian sparkling wine (SW). Dose of SW was 150 ml ones a day; total amount of consumed SW was 2,635 ± 463 ml for 17.6 ± 1.6 drinks.

Complex investigations of patients were performed twice during recuperative sanitaria treatment: at the beginning and at the end of course. The 47 parameters were monitored, including clinical, laboratory and functional data and special tests of stress level. The Reeder test was used to evaluate the psychological stress, the Garkavi blood test was used to evaluate the morphological stress. The parameter dynamics (Δ) was calculated as follows:

Δ = value at the beginning of treatment − value at the end of treatment

Statistical analysis of obtained data (Student's t-test and correlation analysis) was performed by "Statistica v.8.5". $P < 0.05$ was considered statistically significant.

24.3 Results

As a result of recuperative treatment, the positive dynamics of health parameters was observed in a majority of patients with AH, CB and CFS. The number of parameters with positive dynamics was more in the A groups (with wine consumption) than in the B groups (control). Table 24.1 presents mean values of parameters that have been reliably changed by the end of treatment.

The correlation analysis showed statistically significant correlation between daily dose of wine and the following parameters: (i) negative dynamics of feeling and complaints on high disposition to sweating, (ii) positive dynamics of segmented leukocyte and lymphocyte content in peripheral blood, and the erythrocyte sedimentation speed in men, (iii) normalizing of elevated cholesterol blood level, and (iv) negative dynamics of bilirubin content in blood plasma, in the AH patients; (v) positive dynamics of complaints on the increased fatigue, inability to relax, irritability, low spirits, sensorial-motor sluggishness, low concentration of attention, low self-esteem and low self-reliance, poor memory, muscle ache and asthenia, dispepsia, low endurance, low libido, morning tension, anxiety, (vi) positive dynamics of psychic and somatic stress severity, basic leukocyte content in peripheral blood, Kerdo index, minute blood volume, and right carotid artery rheo-encephalo-graphic index, in the CFS patients; (vii) negative dynamics of complaints on headache, and (viii) positive dynamics of total amount of all complaints, in the patients with either AH or CFS.

The above mentioned wine effects have a very good compliance with PC effects [5, 9, 10, 12–15]. Some other effects probably are due to other biologically active components of wine [14].

24.4 Conclusion

The obtained results revealed that the most positive wine effect is a successful coping with stress. Statistically significant changes were also observed in psychological and morphological reactions, including positive dynamics in patient's self feeling, various complaints, adaptation and stress reactions (Garkavi test), diastolic blood

Table 24.1 Mean values of the parameters that have been reliably changed at the end of treatment in groups A and B ($p < 0.05$)

	AH		CB		CFS	
Parameters and units	A	B	A	B	A	B
Positive dynamics of patients feeling (points)	0.7*†	0.4*†				
Positive dynamics of systolic arterial pressure (mm Hg)			7.8*			
Positive dynamics of diastolic arterial pressure (mm Hg)	8.7*†	4.6*†	5.1*			
Positive dynamics of right carotid artery rheo-encephalographic index					0.2*	
Positive dynamics of Robinson index			12.4†	7.6†		
Positive dynamics of Gentch test (s)			−6.0†	−2.2†		
Dynamics of hemoglobin content (g/l)			1.8† (negative)	−1.1† (positive)		
Frequency of normalizing of previously increased cholesterol content (more than 5 mol/l at the beginning of treatment)			0.2†	0.0†		
Basic leukocyte content in peripheral blood (%)					0.3†	0.0†
Positive dynamics of Garkavi test (points)	–0.2†	–0.3*†				−0.1*
Treatment efficacy (points)	1.0†	0.8†				
Frequency of disappearance of desire to drink strong alcohol beverages	0.1†	0.0†	0.3†	0.0†	0.3†	0.0†
Frequency of undesirable side wine effects	0.1†	0.0†	0.2†	0.0†	0.3†	0.0†

Note: †Statistically significant difference in the parameter changes at the end of treatment between A and B groups; *statistically significant difference in the parameter changes from the beginning to the end of treatment within the group

pressure, minute blood volume, right carotid artery rheo-encephalo-graphic index, Robinson index, Kerdo index and Gentch test.

About quarter of patients had noted the attenuation of desire to drink strong alcohol beverages.

The results are in good accordance with the previously observed effects of grape's wines on prevention of stress-induced diseases, especially, on cardiovascular diseases.

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Chapter 25
Wine Components Normalize the Cytochrome P450 Content in the Liver and Kidneys of Rats Under Neurogenic Stress

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Abstract A majority of organic environmental contaminants need to undergo the metabolic activation to exert their biological effects (toxic, mutagenic, transforming, etc.). This activation is catalyzed by monooxygenase system, which functional element is cytochrome P450 superfamily. One of the important properties of monooxygenase system is the ability of induction under the influence of external stimuli. Xenobiotics, different physiological factors, and stress can act as these stimuli. In our work, we studied cytochrome P450 content in the organs of the experimental animals under acute neurogenic stress and under the influence of alcohol and grape polyphenols. The obtained results showed that stressed animals had the low liver cytochrome P450 content compared with intact animals. Ethanol administration caused increasing of cytochrome P450 content in the liver of intact and stressed rats. Polyphenolic concentrate Enoant normalized the cytochrome P450 content in liver, whereas grape wines Cabernet and Rkatsiteli significantly increased cytochrome P450 content in both intact and stressed animals. The prophylactic administration of polyphenol concentrates to control animals did not significantly influence the cytochrome P450 content in hepatocytes and in rat kidneys. The cytochrome P450 content significantly decreased in animal kidneys under emotional-pain stress, but this effect was attenuated by ethanol administration. Incubation of isolated hepatocytes in the presence of phenacetin was accompanied by increased levels of cytochrome P450 in the cells. Grape polyphenols reduced cytochrome P450 content in the cells in dose-dependent and environment (e.g. stress, ethanol administration)-dependent manners, which suggests their possible role in the cancer prevention.

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25.1 Introduction

Cytochrome P450 isoenzymes (CYPs) are a superfamily of hemoprotein enzymes localized in the endoplasmic reticulum membrane. They are responsible for catalyzing the metabolism of a great number of endogenous and exogenous compounds. CYPs are also known as mixed function oxidases and mono-oxygenases since metabolism of a substrate by a CYP consumes one molecule of the molecular oxygen and produces an oxidized substrate and another molecule of oxygen appears in water as a byproduct [1]. CYPs are also called polysubstrate monooxygenases since one isoenzyme can have multiple substrates [2]. These enzymes are responsible for biotransformation of drugs and are the body's defense against xenobiotics along with P-glycoprotein. P-glycoprotein is the efflux pump or a transporter present in the brain capillary endothelial cells, intestinal mucosal, renal and tubular cells, hepatic canalicular cells and it is responsible for extrusion or efflux of drugs, thereby enhancing drug elimination.

CYPs are predominantly present in the liver, but are also found in the intestine, lungs, kidneys, brain, etc. Twenty-one families, 20 subfamilies and 57 genes have currently been described in humans. Among them, CYP 1, 2 and 3 account for 70 % of the total hepatic CYPs content and are responsible for 94 % of the drug metabolism in the liver [3]. They are insoluble proteins bound to the endoplasmic reticulum, with complex mechanistic and structural features. However, the first crystal structures of mammalian CYP enzymes, namely CYP2C5, CYP2B4, CYP2C9 and CYP3A4, have recently been determined [4, 5] and, thus, much progress can be expected in this area in near future. It is believed that 15–20 different CYP enzyme isoforms contribute to the drug metabolism in the human liver. However, the CYP enzymes 1A2, 2C9, 2C19, 2D6, 2E1 and 3A4 are considered the most important among them [6–8].

To identify which CYPs are responsible for metabolizing drugs, several *in vitro* approaches have been developed. Some of these approaches include: (i) metabolism by microsome derived from cDNA-expressed enzyme, (ii) use of selective inhibitors with microsomes, (iii) immunoinhibition of CYP by isoforms specific anti-P450 antibodies in microsomes and (iv) correlation of the drug candidate metabolites formation with several isoform specific P450 activities in a panel of liver microsomes [9]. A combination of approaches is typically required to identify accurately which CYP is responsible for metabolizing a drug. However, the use of cDNA expressed CYPs for the preliminary determination of the principal CYPs involved in the drug candidate's metabolism is a reasonable starting point in a drug discovery.

The most common form of drug interactions entails a foreign chemical acting either as an inhibitor or an inducer of the CYP enzyme isoform responsible for metabolizing an administered drug, subsequently leading to an unusually slow or fast clearance of the drug. More rarely, enzyme stimulation can occur where direct addition of one compound enhances the rate of the reaction for a substrate [10]. Inhibition of the drug metabolism will result in elevation of its concentration in

tissues. This leads to various adverse reactions, particularly for drugs with a low therapeutic index. Constant research in this field has been successfully kept updated by developing web based databases for reported and likely drug candidate interactions. The induction of a CYP enzyme isoform responsible for the metabolism of a drug can reduce its expected therapeutic ability due to depletion of its plasma concentration. Therefore, a higher dose of the parent drug is required for effective therapy, with further dose adjusting as and when the inducer effects are withdrawn. This is true in many instances. It should be noted that CYP enzyme induction rarely leads to toxicity, except in cases where the metabolite is particularly harmful. For example, CYP1A1 and 1A2 have been implicated in increased carcinogenic activation of chemicals. Thus, they are considered as a potential risk factor in certain cancers and, hence, drugs that induce these reactions are preferentially avoided by the pharmaceutical industry [11, 12]. Several popular herbs have been reported to participate in interactions with drugs leading to clinically relevant drug adversities.

A majority of serious cases of drug interactions are resulted from the interference of the metabolic clearance of one drug by yet another co-administered drug, food or natural product. Gaining mechanistic knowledge towards such interactions has been accepted as an approach to avoid adverse reactions. The inductions and inhibition of CYP enzymes by natural products in the presence of a prescribed drug has led to adverse effects. Herbal medicines such as St. John's wort (*Hypericum perforatum*), garlic (*Allium sativa*), piperine (from *Piper* sp.), ginseng (*Ginseng* sp.), gingko (*Gingko biloba*), soya beans (*Glycine max*), alfalfa (*Medicago sativa*) and grapefruit juice show clinical interactions when co-administered with medicines. Plant polyphenols inhibit several isoforms of CYPs [13, 14], potentiate the antioxidant ability of the liver [15], act as a scavenger of oxygen free radicals [16], inhibit the synthesis of pro-inflammatory cytokines and enhance apoptosis.

Resveratrol, which is present in concentrations of about 10 μM in red wine, has been found to inhibit events associated with tumor initiation, promotion and progression. The mechanism involved could be the inhibition of activities catalyzed by CYPs, which activate procarcinogens.

A number of naturally occurring flavonoids have been shown to modulate the CYP system, including the induction of specific CYP isozymes, and the activation or inhibition of these enzymes. Some flavonoids alter CYPs through binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acting as either AhR agonists or antagonists. Inhibition of CYP enzymes, including CYP 1A1, 1A2, 2E1 and 3A4 by competitive or mechanism-based mechanisms also occurs. Flavones (chrysin, baicalein, and galangin), flavanones (naringenin) and isoflavones (genistein, biochanin A) inhibit the activity of aromatase (CYP19), thus decreasing estrogen biosynthesis and producing anti-estrogenic effects being important in breast and prostate cancers.

However, there is not a lot of research in this field yet. In our work, the CYP content in the organs of the experimental animals under the acute neurogenic stress and the influence of alcohol and grape polyphenols on this parameter were studied.

25.2 Material and Methods

Purebred male rats of 180–220 g of the body weight were used in the experiments. Animals were kept on balanced feeding in the conditions of vivarium. Animals daily consumed *per os* low alcohol beverages from grapes of red and white grades for 14 days. These beverages were administered in the active dose of 9 mg of polyphenols/100 g of the body weight. Considering the fact that in the beverages investigated the polyphenol content was quite low to achieve the active dose these beverages were given three times a day by 2 ml of liquids per 100 g of the animal body weight. Ethanol was administrated for 14 days by 40 mg per 100 g of the animal body weight (long-term administration) and 400 mg per 100 g of the animal body weight (acute administration). Control animals received the corresponding volume of the physiological solution.

Stress was caused by immobilization of the rats on the abdomen for 3 h [17]. Animals were decapitated in 3 h after the immobilization. The liver was perfused by the cold extraction medium (0.25 M sucrose in 0.025 M Tris-HCl, pH 7.5). All manipulations with animals were held under chloralose-urethane anesthesia. This study was approved by Animal Use and Care Administrative Advisory Committee of the National University of Pharmacy.

Microsomes were prepared from the liver and kidneys, immediately after sacrificing the rats [18]. The total CYP content was determined according to the method of Omura and Sato [19]. The concentration of hepatic microsomal protein was determined according to the method of Lowry et al. [20].

Hepatocytes were isolated by the two-step collagenase perfusion method [21], and cell viability was determined by trypan blue exclusion. The hepatocyte suspension was diluted to 10^6 cells/ml in the serum-free, supplemented Williams' medium E (containing 1 μM insulin, 100 nM DEX, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) and a 3-ml volume was plated onto culture dishes. Cells (10^7/ml) were incubated in the Eagle medium containing 10 % fetal calf serum, 100 units/l streptomycin, 100 units/l penicillin, 13 mg/ml gentamycin, 100 μM phenacetin and/or 100 μM Enoant, 100 μM grape seeds extract in 95 % O_2/5 % CO_2 atmosphere at 37 °C for 24 h. The reaction was stopped with cold ethanol. Cells were mixed and centrifuged. The content of acetaminophen in the supernatant was determined by the method described elsewhere [22].

All data were analyzed for statistical significance with SPSS 13.0 software. Data were presented as means ± standard deviation. Statistical analysis used one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

25.3 Results

The content of the liver CYP in stressed animals is reduced by 23 % compared to intact animals. CYP is inducible enzyme, which content depends on the animal age and sex, and it also is changed under the influence of external physical, chemical,

Table 25.1 Effect of grape polyphenolic complexes and grape wines on the CYP P450 content in the liver and kidneys of rats with the neurogenic stress, n = 6

	CYP P450 content, nmol/mg protein	
Experimental conditions	Liver	Kidneys
Intact	0.131 ± 0.011	0.064 ± 0.009
Stress	0.117 ± 0.017*	0.037 ± 0.002*
Ethanol (long-term administration)	0.189 ± 0.010*	0.101 ± 0.008*
Stress + Ethanol	0.174 ± 0.017*	0.092 ± 0.017*
Stress + Enoant	0.143 ± 0.018†	0.067 ± 0.018
Stress + Grape seeds extract	0.145 ± 0.011†	0.090 ± 0.009
Stress + Cabernet	0.165 ± 0.012*†	0.069 ± 0.009
Stress + Rkatsiteli	0.166 ± 0.015*†	0.076 ± 0.010
Enoant	0.135 ± 0.005†	0.060 ± 0.005
Grape seeds extract	0.129 ± 0.009†	0.062 ± 0.004
Cabernet	0.157 ± 0.014*	0.073 ± 0.004*
Rkatsiteli	0.159 ± 0.008*	0.078 ± 0.006*

Note: the data presented as mean ± SD, *$p \leq 0.05$ vs. intact animals; †$p \leq 0.05$ vs. stressed animals

and biological factors. The neurogenic stress causes the increased formation of reactive oxygen species (ROS) in the liver, which contributes to the development of the oxidative stress [23].

The development of the oxidative stress can lead to decrease in CYP gene expression, particularly its isoform 2E1, which is involved in the metabolism of ethanol [24]. This is a specific cell response to intensification of peroxidation processes and a shift in the homeostasis in the oxidative direction.

The CYP content in the rat liver is decreased under long-term administration of alcohol (Table 25.1). Similar changes were observed when alcohol was administered into the stressed animals. Currently, there is a significant amount of literature data pointing at the ethanol involvement in the CYP regulation. A single dose of ethanol can cause inhibition of CYT 2E1, whereas the chronic ethanol administration may cause induction of CYT 2E1 [25]. These results were obtained not only in laboratory animals, but also in patients with alcoholism [26]. There are also data on induction of CYT 2A5 under the influence of ethanol [27]. However, it should be noted that the activation of CYP 2E1 is the key step of this process.

The phenomenon of CYP induction is an important component of the adaptive response to xenobiotics that enter the cell. Certain xenobiotics can induce the enzymes of their own metabolism, e.g., the effect of substrate induction, which maintains detoxification functions of the body and further excretion of xenobiotics.

Prophylactic administration of polyphenolic concentrates Enoant, grape seeds extract and wines Cabernet and Rkatsiteli for 14 days resulted in the increase in the CYP content of the liver (Table 25.1). Noteworthy, Enoant normalized the content of the enzyme, while grape wines Rkatsiteli and Cabernet significantly increased the content of CYP not only comparing to the stressed animals, but also to the intact control.

Prophylactic administration of polyphenolic concentrates in animals had no effect on the CYP content in the liver cells (Table 25.1). These data correspond to the literature data that the introduction of red wine stimulates the blood plasma antioxidant activity, whereas the complex of grape polyphenols, which does not contain alcohol, reduces the content of CYP [28].

The CYP content normalization in the liver may be associated not only with the antioxidant action of polyphenols of grapes and grape wines. The CYP increased content after the prophylactic administration of polyphenols may be the result of the polyphenols ability to induce the gene expression responsible for CYP synthesis.

The similar tendencies were observed under the study of the CYT content in the rat kidneys (Table 25.1). It should be noted that the CYP content is lower in the kidney tissue. Under the neurogenic stress, the CYP content is significantly decreased in animal kidneys, but these changes are reversed by the ethanol administration. The ethanol administration to the control group increased the content of CYP. This is in agreement with the literature data [29].

Since the content of CYP in the tissues correlated with the development of the oxidative stress, normalization of the enzyme content may be due to the antioxidant activity of grape polyphenols. A higher content of CYP (compared to intact animals) in case of prophylactic administration of Cabernet and Rkatsiteli to the control group may be caused by the wine polyphenols only partially blocking the effects of ethanol.

Unsubstituted flavone and flavonoids without hydroxyl groups in the benzene ring A induce CYPs (1A1, 1A2, 1B1, 2E1) [30]. At the same time, these substances induce the biosynthesis of enzymes participating in the second phase of detoxification, such as glutathione transferase and UDP-glucuronyl transferase, and providing the final detoxification and excretion of hydrophilized toxins from the body. For example, flavones tangerine and nobeletin increase the benzpyrene hydroxylase activity.

Mechanisms of regulation of the CYP activity are poorly understood. It is thought that its induction may be mediated by binding to the AhR [31]. Increased interaction between the CYP and NADPH-reductase is also possible. However, the information considering the induction mechanisms is limited by several members of the CYP family, but the transcriptional activation mechanism is described for the largest part of subfamilies. It is known that gene activation of these enzymes involves different nuclear receptors, which interact with the xenobiotic ligands and are translocated from the cytoplasm to the nucleus, or aggravated in the nucleus, where they form heterodimers with their nuclear partners and interact with the target genes.

The necessary step in the initiation of tumor formation under the action of environmental carcinogenesis is the enzymatic formation of the final carcinogen from chemically inert compounds. This process requires one or several steps, but the reactions of oxidation in the monooxygenase system, which basic functional enzyme is CYP, are essential. There is some evidence that genistein and daidzein are inducers of CYPs 1A1, 1A2, and 1B1, and inhibit the poly(ADP-ribose)-polymerase, an enzyme required for DNA repair, activity in the rat liver [32].

Changing the ratio of non-protein signaling molecules in the CYP induction is one of the possible mechanisms of carcinogenesis promotion. The long-term

Table 25.2 The effect of Enoant administration in different doses (in ml/100 g body weight) and in combination with long-term ethanol administration on the liver CYP content in rats with the neurogenic stress, n = 6

Experimental conditions	CYPe P450 content, nmol/mg protein
Intact	0.137 ± 0.002
Stress	0.102 ± 0.004*
Ethanol (long-term administration)	0.186 ± 0.010*
Stress + Ethanol	0.162 ± 0.015*†
Stress + Ethanol + Enoant(0.01)	0.118 ± 0.005*
Stress + Ethanol + Enoant(0.03)	0.121 ± 0.010*
Stress + Ethanol + Enoant(0.05)	0.131 ± 0.010†
Stress + Ethanol + Enoant(0.07)	0.142 ± 0.010†
Stress + Ethanol + Enoant(0.10)	0.118 ± 0.006*†
Stress + Ethanol + Enoant(0.15)	0.114 ± 0.006†

Note: the data presented as mean ± SD, *$p \leq 0.05$ vs. intact animals; †$p \leq 0.05$ vs. stressed animals

Table 25.3 Effect of Enoant on the liver CYP content in rats under acute ethanol administration, n = 6

Experimental conditions	CYP P450 content, nmol/mg protein
Intact	0.137 ± 0.002
Ethanol (acute administration)	0.114 ± 0.010*
Ethanol + Enoant (dose 0.07)	0.142 ± 0.015*†

Note: the data presented as mean ± SD, *$p \leq 0.05$ vs. intact animals; †$p \leq 0.05$ vs. ethanol

administration of CYP inductors, which is necessary for promotion, leads to "unphysiological" conditions in the cell: the highly active enzymes function for a long time, while under "physiological" conditions they manifest the short-term activity. As a result, the newly synthesized CYPs need the substrates for oxidation, but the lack of xenobiotics leads to oxidation of endogenous compounds that changes the natural balance of signaling molecules [32]. At the same time, this oxidation processes stimulate the formation of highly active oxygen-containing metabolites, sucg as epoxides, peroxides, alcohols.

At the next stage of our experiments, we studied the effect of different doses of Enoant on the CYP content. As can be seen from Table 25.2, administration of alcohol significantly increased the CYP content in the stressed animals. Prophylactic administration of Enoant together with alcohol revealed its dose-dependent effect. Thus, in the dose of 0.07 ml per 100 g of body weight, Enoant normalized the CYP content. Since ethanol can inhibit CYP gene expression, the results obtained may indicate the possible stimulatory effect of Enoant polyphenols on this process. A single administration of high doses of ethanol leads to a decrease in CYP content in rat liver (Table 25.3). These results are consistent with literature data [25]. Introduction of Enoant normalizes effect on this parameter. Thus, these data indicate that Enoant normalizes the content of CYP under the oppression and the amplification of its synthesis by ethanol administration (Tables 25.2 and 25.3).

Table 25.4 Effect of polyphenol concentrate Enoant on the phenacetin-induced CYP synthesis in rat hepatocytes, n = 6

Experimental conditions	CYP P450 content, nmol/mg protein	Acetaminophen content, nmol/mg protein
Intact cells	0.348 ± 0.045	–
Phenacetin	0.668 ± 0.079*	967 ± 67
Phenacetin + Enoant	0.491 ± 0.051*	345 ± 43†
Phenacetin + Grape seeds extract	0.510 ± 0.055*	472 ± 52†
Enoant	0.449 ± 0.050*	–
Grape seeds extract	0.476 ± 0.050*	–

Note: the data presented as mean ± SD, *p ≤ 0.05 vs. intact cells, †p ≤ 0.05 vs. phenacetin

It is known that various polyphenols have different effects on the synthesis of CYP [30, 32]. Incubation of isolated hepatocytes in the presence of phenacetin was accompanied by increase of CYP content in the cells (Table 25.3). After phenacetin penetrates into the cells, it is metabolized by CYP P450 to acetaminophen, most of which enters into the conjugation reaction with glucuronic acid to form inactive metabolites. In addition, it has been found that phenacetin is a powerful inductor of CYP1 A2 synthesis [33].

Xenobiotic metabolism is divided into two phases. In phase I, enzymes, e.g., CYP P450 oxidase, introduce reactive or polar groups into xenobiotics [34]. These modified compounds are then conjugated to polar compounds in phase II. These reactions are catalyzed by transferase enzymes. In subsequent phase II, the activated xenobiotic metabolites are conjugated with charged species, such as glutathione, sulfate, glycine, or glucuronic acid [35]. These reactions are catalyzed by a large group of transferases with a broad specificity, which in combination can metabolize almost any hydrophobic compound that contains nucleophilic or electrophilic groups. In the second phase of biotransformation, hydrophilicity of metabolites is increased and this facilitates their excretion by the kidneys.

It has been found that, in 80–90 % of cancers, chemical and environmental factors are involved. Moreover, the vast majority of carcinogens are formed in humans and animals from pro-carcinogens involving mainly CYT P450, especially, the I family, i.e. CYP1A1, CYP1A2, and CYP1B1. As a result of the CYP-dependent metabolism, intermediates that often exert toxicity or carcinogenicity are formed. At the same time, they are also targets for phase II enzyme-dependent conjugation reactions transforming them into inactive polar products suitable for excretion by the kidneys. In some uncommon cases, the phase II-dependent metabolism could produce more harmful products than the parent compounds. Many different cytotoxic drugs are inactivated by the action of CYP, whereas several pro-drugs are activated by the action of CYP, rendering them cytotoxic and effective in cancer chemotherapy [36]. The fact that grape polyphenols reduced the CYP content in the cells and reduce the formation of acetaminophen suggests that they manifest the anti-carcinogenic effect (Table 25.3).

During the incubation of isolated hepatocytes in the presence of Enoant and grape seeds extract, an increase in the level of CYP is observed, but less significant than the effect of phenacetin (Table 25.4).

It is known that flavonoids containing hydroxyl groups in the ring A are CYP inhibitors, and flavonoids that do not contain them are activators (flavon, tangerine, nobiletin) [37]. For example, quercetin, kaempferol, naringenin (containing 2 hydroxyl groups in the ring A) inhibit CYP 3A4, which metabolizes nifedipine and filodipin, the dihydropyridine calcium channel blockers. There are some published results proving the inhibitory effect of gallic acid on the CYP expression.

At the same time, the organ and species specific expressions of different enzyme isoforms have been described, which, in turn, determines the organ and species specific responce to different polyphenols. Thus, genistein and daidzein, which stimulate the CYP 3A11 expression in the hepatocytes, inhibit the CYP 324 V expression in mice enterocytes [32]. This fact is important for prevention and treatment of colon cancer. Curcumin inhibits the CYP 1A1 and 1B1 expression induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in the kidney [38, 39].

25.4 Conclusion

CYPs are the key enzymes in cancer formation and cancer treatment. They mediate the metabolic activation of numerous precarcinogens and participate in the inactivation and activation of anticancer drugs. Since all CYPs that metabolize xenobiotics are polymorphic, much emphasis has been put on the investigation of a relationship between the distribution of the specific sample CYP alleles and the risk for different types of cancer, but a consistent view does not yet exist. This is to a great extent explained by the fact that the CYPs involved in activation of precarcinogens are in general not functionally polymorphic. This is in contrast to CYPs that are active in drug biotransformation where large inter individual differences in the capacity to metabolize therapeutic drugs are seen as a consequence of polymorphic alleles with altered function.

The results suggest that the grape polyphenols exerted different effects on the CYP content. These effects are dose dependent, and also are depended on the type of experimental design (stress, ethanol administration). Increased CYP expression is the important factor in the inactivation of toxins and xenobiotics, while inhibiting the CYP expression is the necessary stage in the carcinogenesis prevention.

The organ and species specific expression of different enzyme isoforms, and obtained results considering the specificity of the grape polyphenols action on the liver and kidneys warranted an investigation of the grape polyphenols influence on content, expression and activity of CYP. The findings suggested that grape polyphenolic concentrates and wine have potential as a chemopreventive agent.

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